NON-INVASIVE MEASUREMENT OF OXIDATIVE STRESS IN SENIOR AND EXERCISING HORSES.

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ABSTRACT

The published data regarding oxidative stress in horses is generally concerned with blood and muscle samples. The aim of this thesis was to use non-invasive markers as a novel approach to investigate cellular stress in performance and senior horses. In addition, urine was analysed using ^{1}H NMR spectroscopy and renal insult during ageing and exercise was investigated using urinary N-acetyl-beta-D-glucosaminidase (NAG) activity.

The results demonstrated that urinary thiobarbituric acid reactive substances (TBARS) in horses could be measured using a method adapted from Yagi (1976) and were significantly decreased following supplementation with antioxidants in the form of dandelion and milk thistle ($p<0.05$). Urinary TBARS were seen to decrease in horses supplemented with vitamin E at a level of 4mg/kg bodyweight, but subsequently were unaffected in horses performing a sub-maximal exercise test on a treadmill when supplemented with vitamin E at a level of 3 mg/kg bodyweight.

Urinary TBARS were seen to increase with age ($P<0.05$), consistent with increased lipid peroxidation in senior horses. Analysis using ^{1}H NMR spectroscopy revealed higher levels of aromatic amino acids in the urine of senior horses ($p<0.05$) and proteinuria quantification using the biuret assay demonstrated increased total proteinuria in the urine of senior horses compared to young horses ($p<0.05$). This indicated that subtle changes in renal, hepatic and endocrine functions may be evident in senior horses.

TBARS in equine sweat could be measured using a method adapted from Yagi (1976) and were significantly decreased in horses performing a sub-maximal exercise test on a treadmill, following vitamin E supplementation ($p<0.05$). In addition, free radical scavenging activity of equine saliva could be measured using a method adapted from Atsumi *et al* (1999). Urinary NAG activity proved to be difficult to measure in the horse and may require further investigation to establish its potential use as a marker of renal insult in horses.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 EQUINE NUTRITION

In Great Britain there are an estimated 900,000 horses and over 2 million riders (www.beta-uk.org). The majority of sports horses are competing in jumping, dressage and eventing and there are approximately 14,000 racehorses in training (www.bhb.co.uk).

Horses are non-ruminant herbivores and have evolved to eat high fibre diets, which are fermented by anaerobic organisms present in the caecum and colon. The domesticated horse consumes a variety of feeds ranging from long stemmed forage to cereals with large amounts of starch. Starch is digested to glucose in the small intestine by pancreatic alpha-amylase and other soluble carbohydrates are degraded to their component sugars by alpha-glucosidases. Fats are digested efficiently in the small intestine and are a valuable source of energy for working horses. Fibre digestion in the large intestine results in the production of volatile fatty acids, namely acetic acid, butyric acid and propionic acid. These can be used for the production of ATP or fat synthesis and propionic acid can be converted to glucose in the liver.

Knowledge of horse nutrition has increased dramatically over the last 20 years but huge gaps still exist in the understanding of how nutrition affects exercise performance (Pagan 1999).

The most important consideration when feeding horses for athletic performance is the provision of energy, where chemically bound energy from feed is converted into mechanical energy for muscular movement. Other nutrients are necessary for the optimum function of physiological systems used during exercise and a deficiency of any nutrient may impair athletic performance (Pagan 1999). For example, glycolytic kinases require magnesium for full activity and iron and copper are electron carriers in oxidative phosphorylation. Zinc is an integral part of carbonic anhydrase, needed for carbon dioxide transport from muscles to the lungs (Fogelholm 1992) and thiamin and biotin act as cofactors for enzymes regulating glycolysis, tricarboxylic acid (TCA) cycle and amino acid degradation. Riboflavin, niacin and panthothenic acid also participate in glycolysis and the TCA cycle as well as in oxidative phosphorylation, β oxidation and amino acid degradation.

There is very little direct evidence on the vitamin and mineral requirements of the horse and some recommendations are based largely on experience with other animals. In certain circumstances the intake of vitamins and minerals may fall below basal requirements, for example, where poor quality or minimal amounts of forage is fed or where horses are fed unsupplemented cereals (straights) instead of compound feed. Unsupplemented cereals do not provide adequate levels of vitamins and minerals for horses expected to perform to their genetic potential (Lucas 1999). For example, the

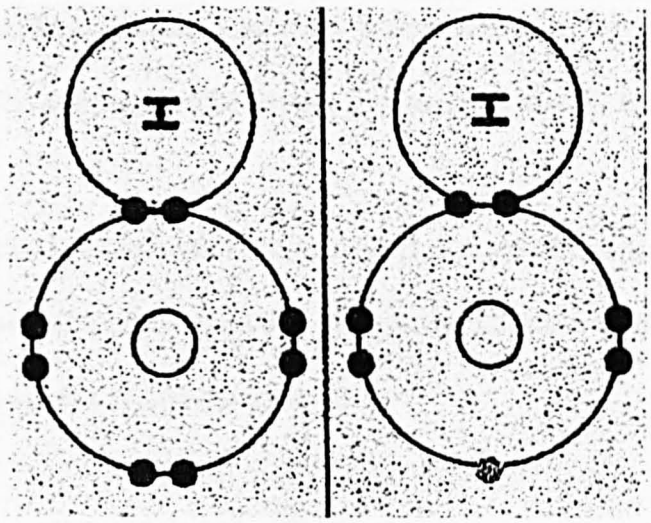
NRC (National Research Council) recommendation for vitamin E intake is 80mg/kg DM and hay and cereal grains contain less than 50mg vitamin E per kilogram of dry matter (Lawrence 1994), therefore supplemental vitamin E must be added to rations to meet the NRC recommendation.

In addition to basic nutrient requirements consideration is also given to nutritional ergogenic aids. An ergogenic aid is a substance that can increase or improve work production (Lawrence 1994). Antioxidant nutrients are thought to enhance performance by aiding recovery because of their ability to detoxify free radicals that are produced during strenuous exercise (Applegate 1999) but evidence for positive effects of supplementation of antioxidants to horses, is lacking.

1.2 FREE RADICALS

Atoms are made up from three basic particles, protons, neutrons and electrons. The protons and neutrons are found in the nucleus of an atom and electrons move around the nucleus in distinct energy levels. Usually, in an atom electrons are arranged in pairs. Every electron has a directional spin and the electrons in a pair have opposite spins.

A free radical is any species capable of independent existence that contains one or more unpaired electrons (Gutteridge and Halliwell 1994). The unpaired electrons combine with electrons with opposite spins in other molecules, consequently, free radicals are highly reactive and can damage cells and tissues. Molecular species include hydroxyl, peroxy and superoxide radicals.



A B

Figure 1.1, the hydroxyl ion and the hydroxyl radical illustrating the unpaired electron in the hydroxy radical.

A is the hydroxyl ion (OH-) with 9 protons and 10 electrons

B is the hydroxyl radical (OH•) with 9 protons and 9 electrons

Reactive oxygen species (ROS) and oxygen free radical are not synonymous terms. Unlike the latter, ROS represent a broader spectrum of species including non radical derivatives of oxygen e.g. hydrogen peroxide and singlet oxygen that are capable of inciting and propagating oxidative tissue damage (Sen 1995).

One of the primary sources of free radical production is through the aerobic metabolic pathway. In addition, free radicals are generated from a number of normally functioning enzymes, for example, xanthine oxidase, cytochrome p450, monoamine oxidase and nitric oxide synthase. In the brain, free radicals are produced from the

autooxidation of noradrenaline and dopamine. The oxidation of catechols to quinones generates reduced forms of molecular oxygen, sources of superoxide and hydrogen peroxide.

The sites of free radical generation encompasses all cellular constituents including mitochondria, lysosomes, peroxisomes and nuclear endoplasmic reticular, and plasma membranes as well as sites within the cytosol.

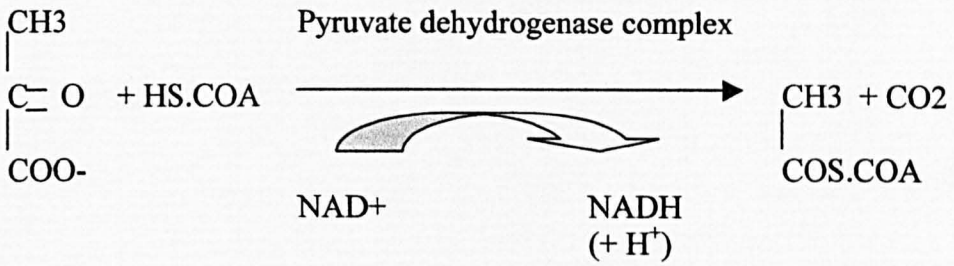
1.3 THE AEROBIC METABOLIC PATHWAY

The intracellular substance used to energise almost all cellular functions is adenosine triphosphate (ATP).

Although the body stores of ATP are not insignificant if horses were to exercise hard for 2-3 seconds they would use all the ATP stored within the cells (Marlin 2002). At the onset of exercise, if muscle contraction is to continue then ATP must be regenerated quickly. This is done by the conversion of ADP to ATP through creatine phosphate catalysed by creatine kinase. However, stores of creatine phosphate are only 2-3 times higher than those of ATP and can only provide energy for a few more seconds of exercise (Marlin 2002).

The aerobic metabolic pathway uses substrates such as glucose and fatty acids to re-synthesise ATP. The initial process, glycolysis, can occur under anaerobic conditions and takes place in the cytoplasm of the cell. During this process glucose is converted to pyruvate. The pyruvate formed from glycolysis is further metabolised to carbon

dioxide and water. The first stage in this process is the oxidative decarboxylation of pyruvate to acetyl coenzyme A in the presence of thiamin diphosphate:



Pyruvate Coenzyme A Acetyl Coenzyme A

The acetyl coenzyme A is then oxidised to carbon dioxide and water via the tricarboxylic acid cycle (TCA cycle). This involves four dehydrogenations, three of which are NAD^+ linked and one FAD linked.

The reduced forms of nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$) deliver electrons from the TCA cycle to the electron transport chain. The electron transport chain consists of several redox catalysts that are located in the inner mitochondrial membrane and it is at this point that oxidative phosphorylation takes place. Electrons flow from hydrogen to oxygen in a transfer reaction to liberate energy for the phosphorylation of ADP to form ATP. As a consequence of this essential biochemical reaction highly reactive oxygen containing molecular species are formed. It has been reported that approximately 2 – 5% of the total oxygen flux through the mitochondria can form superoxide radicals (Goldfarb 1999). The main sites of

superoxide radical production in the respiratory chain are complexes III and I (Lenaz 2001) where production of ATP takes place at the transfer of electrons from cytochrome b to c_1 and from cytochrome a_3 to oxygen.

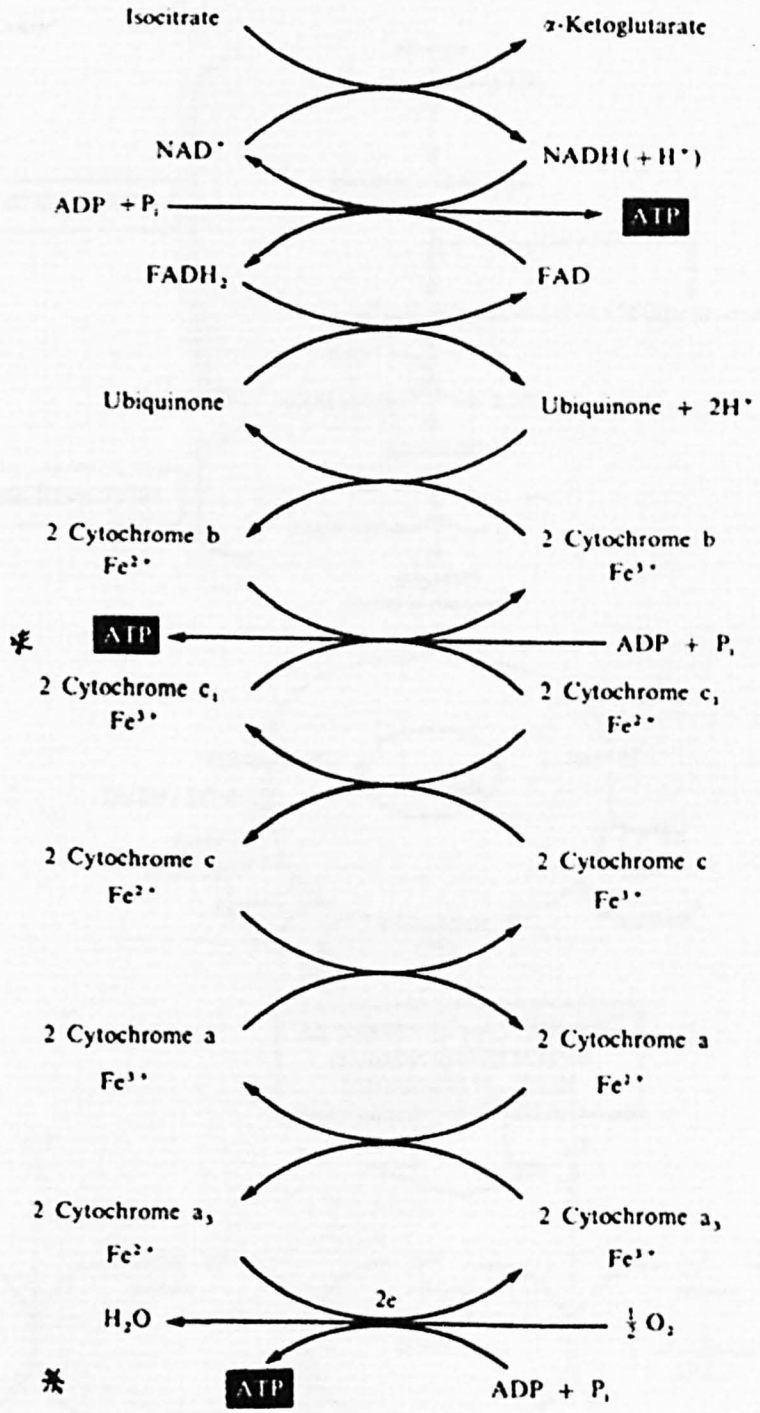


Figure 1.2 Oxidative phosphorylation (McDonald *et al* 1995). * marks the main sites of superoxide generation

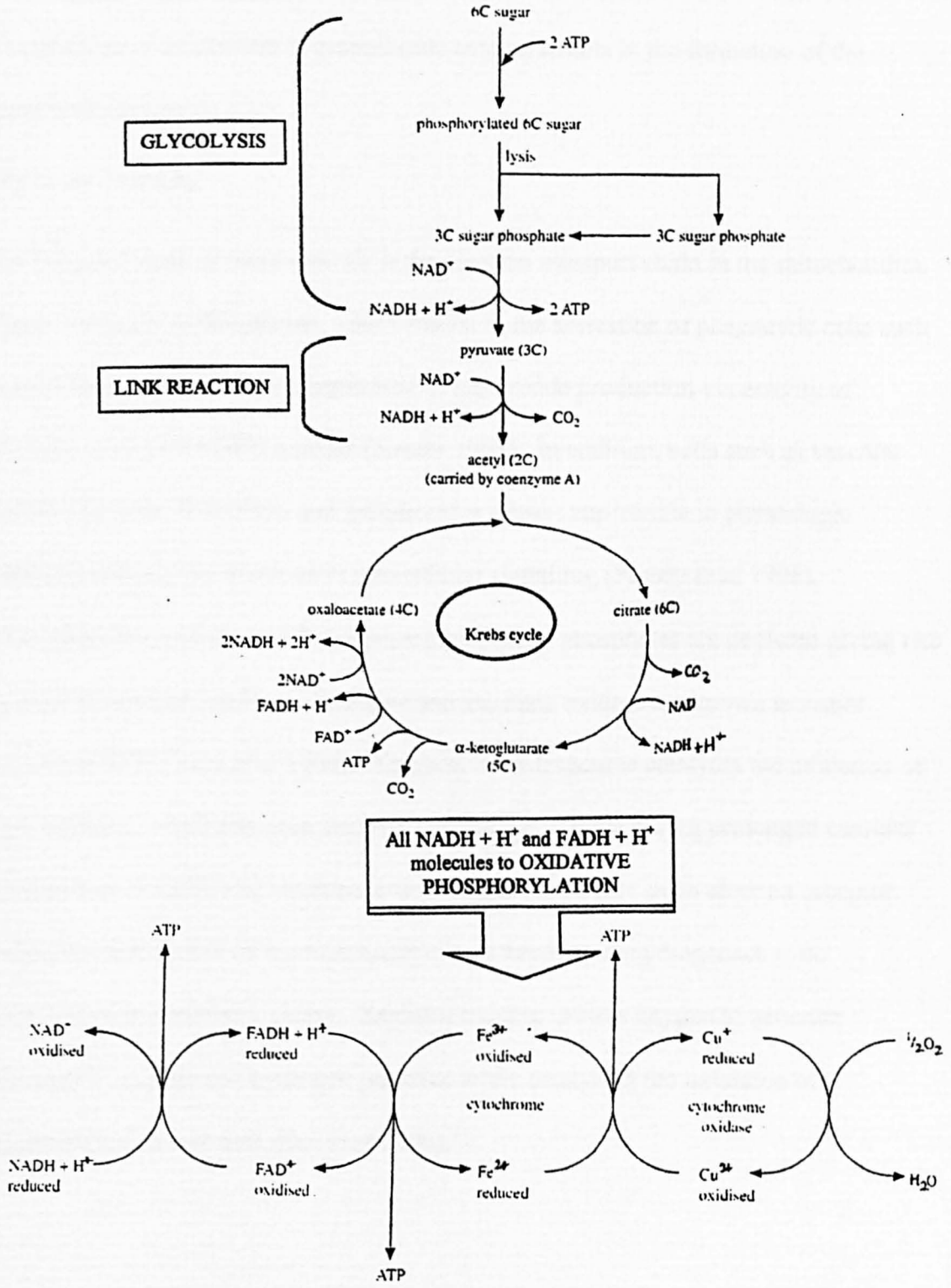


Figure 1.3 Glycolysis, TCA cycle and oxidative phosphorylation (Taylor *et al* 1997)

1.4 Superoxide Radicals

The addition of an electron to ground state oxygen results in the formation of the superoxide structure.

$$O_2 + e^- \rightarrow O_2^{\bullet -}$$

The biggest source of these radicals is the electron transport chain in the mitochondria. Tissue trauma or inflammation, which results in the activation of phagocytic cells such as neutrophils, has also been implicated in superoxide production via activity of enzymes such as NADPH oxidase (Kanter 1998). In addition, cells such as vascular endothelial cells, fibroblasts and lymphocytes release superoxide in physiologic reactions and may be involved in intracellular signalling (Preedy *et al* 1998).
After anaerobic exercise ATP and other high energy phosphates are depleted giving rise to the formation of xanthine. Xanthine and xanthine oxidase are known as major sources of ROS (Vina *et al* 2000). Xanthine dehydrogenase catalyses the oxidation of hypoxanthine, which has been shown to increase at fatigue during prolonged exercise (Febbraio *et al* 1999) and xanthine to uric acid using NAD+ as an electron acceptor. Ischaemic reperfusion of hypoxic tissue causes xanthine dehydrogenase to be transformed to xanthine oxidase. Xanthine oxidase utilises oxygen to generate superoxide radicals and hydrogen peroxide while catalysing the oxidation of hypoxanthine to uric acid (Sen *et al* 2000).

Oxygen radical generation during ischemia-reperfusion in isolated rat liver found that the predominant oxygen radical in the liver during reperfusion following ischaemia was the superoxide radical (Okuda *et al* 1992).

The superoxide radical is unstable in aqueous solutions because of dismutation reactions leading to non-enzymic generation of hydrogen peroxide. Dismutation is said to occur when the superoxide radical gives up its unpaired electron to another superoxide radical. This leads to the oxidation of the first radical to O_2 and the reduction of the second radical to H_2O_2.

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

(Gutteridge and Halliwell 1994)

In addition superoxide anions can be converted to the highly reactive hydroxyl radicals upon the action of iron (II).

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^-$$

1.5 Hydroxyl Radicals

Hydroxyl radicals ($OH^\bullet$) are the most reactive of all radicals. They can be generated by the **Fenton reaction** where hydroxyl radicals are produced from the reaction of hydrogen peroxide with reduced transition metal ions.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

When hydroxyl radicals react with other biological molecules they cause a radical chain reaction, where less reactive radicals are formed. The types of hydroxyl radical reactions that occur are, hydrogen abstraction, where a hydrogen atom is removed from the biological molecule, resulting in water and another less reactive radical. Addition, where the $OH^{\bullet}$ reacts with the aromatic ring structure of a biological molecule, such as the pyrimidine and purine bases of DNA. This leads to the production of a less reactive radical and the damaging of DNA bases sugars and strands. Electron transfer reactions where an electron is moved from one compound to another, for example,

$$Cl^{-} + OH^{\bullet} \rightarrow Cl^{\bullet} + OH^{-}$$

(Halliwell and Gutteridge 1995).

When two hydroxyl radicals meet they can join together to form hydrogen peroxide

$$OH^{\bullet} + OH^{\bullet} \rightarrow H_2O_2$$

Hydrogen peroxide is not a free radical, as it does not contain any unpaired electrons but it can act as a weak oxidising agent and can therefore cause damage to cells and enzymes. As hydrogen peroxide can generate the hydroxyl radical, through the Fenton reaction it can be regarded as a 'mobile time bomb' (Gutteridge and Halliwell 1994).

1.6 Singlet Oxygen ($^1O_2$)

Oxygen has 12 electrons, two of which in the outer orbit are unpaired and both of which have the same directional spin, as opposed to opposite ones. Oxygen therefore behaves

as a diradical. This form of oxygen is the most stable state of oxygen and is known as the ground state. Singlet oxygen is an excited form of ground state oxygen where it loses an electron to a newly formed outer shell in its structure. This electron is unpaired and so this oxygen state is reactive and can react with most biological molecules.

1.7 Non-oxygen radicals

Although oxygen radicals form the most important group of radicals, in biological systems other radicals may play a role as well. Thiol compounds (R-SH) can be oxidised in the presence of transition metal ions to thiyl radicals ($RS^{\bullet}$).

$$RSH + Cu^{2+} \rightarrow RS^{\bullet} + Cu^{+} + H^{+}.$$

Thiyl radicals are sulphur centred radicals that demonstrate considerable reactivity (de Zwart 1998).

Nitric oxide is produced by various mammalian cells. The nitric oxide radical ($NO^{\bullet}$) can react with $O_2^{\bullet-}$ to form the highly reactive non-radical peroxynitrite, which can, under certain circumstances, spontaneously decompose to form $OH^{\bullet}$ and $NO_2^{\bullet}$ radicals

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^{-}$$

$$ONOO^{-} + H^{+} \rightarrow ONOOH \rightarrow NO_2^{\bullet} + OH^{\bullet}$$

(de Zwart 1998).

Table 1.1 Estimated half lives of reactive oxygen species (Sies and Stahl 1995).

Reactive oxygen species	**Half life**
Hydroxyl radical ($HO^{\bullet}$)	1×10^{-9} s
Alkoxyl radical ($RO^{\bullet}$)	1×10^{-6} s
Peroxyl radical ($ROO^{\bullet}$)	7 s
Singlet oxygen ($^1O_2$)	1×10^{-6} s
Nitric oxide radical ($NO^{\bullet}$)	1 – 10 s
Peroxynitrite ($ONOO^-$)	0.05 – 1 s

1.8 FREE RADICAL DAMAGE

The damage caused by free radicals involves the process of oxidation (any chemical reaction that involves the loss of an electron from an atom) and the actual damage to the tissues is called oxidative stress (Youngston 1994).

Free radicals can damage nucleic acids, protein and free amino acids, lipids and lipoproteins, carbohydrates and connective tissue macromolecules (Larkins 1999).

1.8.1 Lipid peroxidation

Prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Lipid peroxidation is a radical chain reaction that damages polyunsaturated fatty acids (PUFAS), the main component of phospholipid bilayer cell membranes.

Carbon-hydrogen bonds positioned either side of, or in-between carbon-carbon double bonds (C=C), have weaker bonds than those not associated with C=C. Therefore, the numerous unsaturated bonds in PUFA are more prone to radical attack than saturated fatty acids (Noguchi and Niki 1998).

The PUFAS that are mainly at risk from lipid peroxidation are linoleic acid, linolenic acid and arachidonic acid (Oh-ishi *et al* 2000).

Linoleic acid (2 double bonds)

$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$

Arachidonic acid (4 double bonds)

$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$

OH• is often the initiating radical as it has higher bond dissociation energy than most radicals, enabling it to remove a hydrogen atom from methyl groups (Noguchi and Niki 1998).

Table 1.2 Bond Dissociation Energy for Abstraction of a Hydrogen Atom (Noguchi and Niki 1998).

Radical	Bond Dissociation Energy (kcal/mol)
Hydroxyl $HO^{\bullet}$	119
Alkoxyl $LO^{\bullet}$	104
Peroxyl $LOO^{\bullet}$	88

The process of lipid peroxidation is initiated when a free radical (R•) removes a hydrogen atom from a polyunsaturated lipid (LH) generating a carbon-centred free radical (L•). This free radical reacts with oxygen to form a peroxyl radical, LOO•. The peroxyl radical may then remove a hydrogen atom from another PUFA forming a lipid hydroperoxide (LOOH) and another L•. This process can continue until all PUFAs are exhausted unless there is a chain terminating reaction.

Hydroperoxides can be degraded in the presence of redox active metal catalysts forming alkoxyl (LO•) or peroxyl radicals. Such reactive intermediates are capable of further reactions which further propagate lipid peroxidation.

If two free radicals meet and interact then a non-radical species can be formed, terminating the lipid peroxidation process.

$2L\bullet \rightarrow L\bullet L$

$2LOO\bullet \rightarrow LOOL + O_2$

$L\bullet + LOO\bullet \rightarrow LOOL$

$RH + L\bullet \rightarrow R\bullet + LH$

$RH + LOO\bullet \rightarrow R\bullet + LOOH$

(Rosen *et al* 1999).

Compounds such as vitamin E and vitamin C that yield radicals unable to continue the chain propagation step will also terminate lipid peroxidation (Oh-ishi *et al* 2000).

Lipid peroxidation causes changes in the arrangement and structure of the lipid bi-layer that surrounds biological membranes, which may lead to a change in both the membrane's fluidity and permeability (Oh-ishi *et al* 2000).

1.8.2 Damage to DNA

The hydroxyl radical is particularly damaging to DNA as it can attach itself to the purine and pyrimidine bases that are attached to the deoxyribose sugar backbone of the DNA molecule. For example, the hydroxyl radical can attach to the purine base guanine, creating a hydroxyguanine radical which leads to the formation of 8-hydroxyguanine. It is the pairing of the purine and pyrimidine bases that allows DNA to replicate, so when the hydroxyl radical attaches it creates an altered molecule, causing the DNA sequence to be 'misread' leading to a mutation in the DNA strand. This mutation can lead to the creation of mutated proteins which may not be able to perform the task they are meant to.

In addition, DNA can be fragmented. This can occur where $OH^{\bullet}$ formed directly in the nucleus attacks the DNA, or by the production of $OH^{\bullet}$ by the metal ions that are bound to nearby proteins. Intracellular levels of calcium rise in response to oxidative stress and this leads to an increase in nuclease activity which causes DNA fragmentation (Gutteridge and Halliwell 1994).

More oxidative stress is found to occur in mitochondrial DNA than nuclear DNA, which is related to the fact that the electron transport chain occurs in the mitochondria

and so the associated formation of free radicals (Radak 2000) will effect mitochondrial DNA.

1.9 DEFENCE

There are a number of endogenous physiological defence systems used to detoxify ROS. A second line of defence is provided by exogenous antioxidants primarily obtained as nutrients. They work by giving up an electron to the free radical or by giving up a hydrogen atom, leading to the formation of water, for example,

$$H + OH^{\bullet} \rightarrow H_2O$$

Antioxidants may be defined as any substance that, when present in low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, Gutteridge 1989).

Antioxidants are both enzymic and non enzymic. They

a) prevent radical formation
b) scavenge the radical species and convert them to a lesser active molecule
c) bind with the metal ions which convert less reactive free radicals into the more reactive ones
d) repair damage to target cells
e) remove oxygen or decrease local oxygen concentrations.

Endogenous tissue antioxidant enzymes are important in protecting the cell against free radical damage. The primary enzymes are superoxide dismutase, catalase, gluathione

reductase and glutathione peroxidase. Enzyme activity changes following exertion or differences in enzyme activity between physically active and sedentary people have indicated that physical exercise intensifies the activity of human erythrocyte or muscle SOD by 22-83%, catalase by 12-121% and glutathione peroxidase by 28-125% However, results showing no effects of physical exercise have also been reported (Fogelholm 1992).

1.9.1 Superoxide Dismutase (SOD)

SOD protects cells against superoxide radicals. There are two forms, one containing copper and zinc, Cu/Zn-SOD and one containing manganese Mn-SOD. Cu/Zn-SOD is localised in the cytosol and nucleus and Mn-SOD is located in the mitochondrial matrix. Cu/Zn-SOD contains two Cu (II) and two Zn (II) atoms per molecule. Zinc has a structural, stabilising role, while Cu^{2+} is directly involved in the catalytic activity (http://www.worthington-biochem.com/manual/s/SOD).

The enzyme catalyses the dismutation reaction of the superoxide radical to oxygen and hydrogen peroxide.

$$\mathbf{SOD\text{-}Cu^{2+} + O_2^{\bullet -} \rightarrow SOD\text{-}Cu^{+} + O_2}$$

$$\mathbf{SOD\text{-}Cu^{+} + O_2^{\bullet -} + 2H^{+} \rightarrow SOD\text{-}Cu^{2+} + H_2O_2}$$

(www.srs.dl.ac.uk/mbg/sod)

1.9.2 Catalase

Catalase functions to break down hydrogen peroxide into water and oxygen

$$2H_2O_2 \rightarrow H_2O + O_2$$

Catalase is composed of four sub-units and each sub-unit contains a haem group. Haem consists of a protoporphyrin ring and a central iron atom.

Catalase functions by the oxidation of iron within its haem group. It removes an electron from two molecules of hydrogen peroxide to form two water molecules and one oxygen molecule.

Catalase has one of the highest turnover numbers for all known enzymes (40,000,000 molecules/second) (http://crystal.uah.edu/~carter/enzyme/catalase)

1.9.3 Glutathione reductase

Reduced glutathione (GSH) functions to protect cellular proteins from thiol oxidation. When challenged with oxidative stress, intracellular glutathione rapidly oxidises to glutathione disulphide (GSSG).

Oxidised GSSG produced intracellularly may be changed back to reduced glutathione by glutathione reductase, requiring NADPH as a cofactor.

1.10 Nutrients

1.10.1 Vitamin E (alpha tocopherol).

Vitamin E is normally found on the inside of cell membranes, for example, mitochondrial membranes contain 1 molecule of vitamin E per 2100 molecules of phospholipid (Halliwell and Gutteridge 1995). Vitamin E protects the cell against lipid peroxidation by donating a hydrogen atom to the peroxyl radical produced during lipid peroxidation, thus terminating the lipid peroxidation chain reaction. Rate constants ranging between 1×10^4 and 1×10^9 have been reported for the reaction of tocopherols with various peroxyl radicals (Sies and Stahl 1995). Vitamin E is the major lipid-soluble antioxidant protecting lipids against peroxidative damage. With microsomes, low density lipoproteins, hepatocytes or whole organs, the vitamin E content often determines susceptibility to damage by hydroxyl, alkoxyl, peroxyl radicals and singlet oxygen (Sies and Stahl 1995).

The tocopherol radical formed is regenerated by ascorbic acid back to vitamin E.

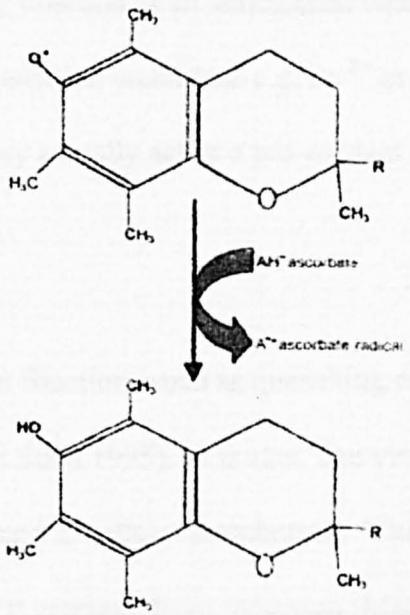


Figure 1.4 Regeneration of α-tocopherol by ascorbic acid

1.10.2 Vitamin C (ascorbic acid)

Vitamin C is a water- soluble vitamin that is found in the cytosol of the cell or in extracellular fluid. It has been shown to react directly with superoxide, hydroxyl, peroxyl radicals and singlet oxygen and terminates the radicals by electron donation. By efficiently trapping free radicals in the aqueous phase before they can initiate lipid peroxidation, ascorbic acid can protect membranes against peroxidative damage. Vitamin C reduces the tocopherol radical and restores the radical scavenging activity of tocopherol, consequently, there is *in vivo* evidence of higher concentrations of vitamin E in tissues of guinea pigs fed high dietary levels of vitamin C (Machlin and Bendich 1987).

However, ascorbate can only function as an antioxidant when transition metal ions are absent. In the presence of transition metal ions e.g. Fe^{3+} or Cu^{2+}, high concentrations of ascorbic acid (~ 1mM) may actually act as a pro-oxidant (Sen 1995).

1.10.3 β carotene

β carotene exerts antioxidant functions such as quenching of singlet oxygen and peroxyl or alkoxyl radicals (Sies and Stahl 1995). It is also, like vitamin E, a lipid soluble antioxidant and has been found in cellular membranes. Vitamin E can protect the conjugated double bonds of β carotene from oxidation (Machlin and Bendich 1987).

1.10.4 Selenium (Se)

Selenium is a trace mineral and is part of the enzyme glutathione peroxidase. Glutathione peroxidase catalyses the reduction of peroxides using glutathione as the reductant. Reduced glutathione (GSH) is oxidised to glutathione disulphide (GSSG)

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O.$$

GSSH is then converted back to GSH by glutathione reductase

$$GSSG + NADPH + H^+ \rightarrow NADP^+ + 2GSH$$

Glutathione peroxidase also reduces lipid hydroperoxides, thus preventing the lipid peroxidation chain reaction.

The functions of Vitamin E and Selenium are closely interrelated. Vitamin E can be regarded as the first line of defence in preventing peroxide formation and the selenium

containing enzyme acting as a second line of defence in destroying any peroxides which are formed before they can damage the cell (McDonald *et al* 1995).

1.11 NON-INVASIVE EXPERIMENTAL TECHNIQUES

In 1954, the Universities Federation for Animal Welfare commissioned a study on humane techniques in experiments with animals. The recommendations were published in 1959 by Russell and Burch and the concepts of replacement, reduction and refinement (the three Rs) were introduced. The three Rs are now accepted concepts within the scientific community and form the basis of existing legislation on animal experimentation.

The principle of 'refinement' is to reduce pain/distress and to improve experimental procedures, e.g. the development of non-invasive methods.

1.12 NON-INVASIVE MEASUREMENT OF OXIDATIVE STRESS

1.12.1 Electron Spin Resonance (ESR)

Free radicals are short lived and are not amenable to direct assay. Free radical activity is usually assessed by indirect methods, such as measurement of the various end products of reactions with lipids, proteins and DNA (Holley and Cheeseman 1993). The only analytical technique that directly measures free radicals is electron spin resonance (ESR) spectrometry.

ESR spectrometry can usually be applied to analysis of samples *in vivo* through the technique of spin trapping. This involves the addition of samples of a compound known as a spin trap, which reacts rapidly with the free radicals to form radical adducts that are more stable and longer lived than the original species.

The technique is usually limited to samples of blood mixed with the spin trap as soon as possible after sampling and therefore would not be a suitable non - invasive technique.

1.12.2 DNA

The number of oxidative hits to the DNA per human cell per day has been estimated to be 10,000 (Sen 1995). A 2 year old rat is estimated to have accumulated 2 million oxidative DNA lesions per cell, which is about twice that in a young rat (Sen 1995). Enzymic excision of oxidative DNA lesions and excretion of such damaged by-products through the urine constitute a crucial step of antioxidant defence.

Vigue *et al* (1993) observed no significant change in the urinary level of the RNA adduct 8-hydroxyguanosine after 90 minutes bicycle exercise in young healthy men. However, the ratio of urinary oxidised nucleosides, adjusted for urinary creatinine, increased 1.3 fold above rest, after 10 hours of marathon running (Allessio and Goldfarb 1988). However, it has been suggested (Collins 1999) that these are unreliable biomarkers of oxidative stress as the markers in urine may result from oxidation of the breakdown products of DNA of dead cells occurring during passage through the kidneys.

1.12.3 Isoprostanes

These are prostaglandin-like compounds and are produced by the reaction of free radicals with arachidonic acid. Both F2 – isoprostanes and their metabolites have been found in urine and have been suggested as potentially useful biomarkers of free radical damage.

Isoprostanes have been measured in plasma and bronchoalveolar lavage fluid of horses 24h before, 1h and 24 h after exercise. Exercise produced a significant increase of isoprostanes in plasma and in bronchoalveolar fluid (Kirschvink *et al* 1999).

Urinary excretion of F2-isoprostanes has been correlated with age and high F2 isoprostanes concentration has been described in diseases such as diabetes (Cracowski *et al* 2000).

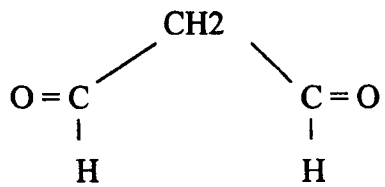
However, the primary source from which the un-metabolised F2-isoprostanes in urine originates is not known. It is uncertain whether they originate from plasma via filtration in the kidney or from local production within the kidney (De Zwart *et al* 1999).

The analytical methods that are available to measure isoprostanes and their metabolites are mainly based on GC MS, however immunoassays are available, specifically validated for human urine, but they are expensive and extensive validation of the assay would be necessary for use in horses.

1.12.4 Lipid peroxidation

The process of lipid peroxidation is often used to confirm the involvement of free radicals in cell damage, as increased lipid peroxidation is consistent with free radical damage.

Polyunsaturated fatty acids (PUFAS) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid peroxides (LOOH). The LOOH and conjugated dienes that are formed can decompose to form numerous other products, including malondialdehyde (MDA). MDA is a volatile low-molecular weight carbonyl formed from PUFAs via primary peroxides.



The structure of malondialdehyde (Halliwell and Gutteridge 1991)

A widely used measure of lipid peroxidation is the thiobarbituric acid (TBA) assay. This involves incubation of samples containing MDA (amongst other lipid peroxidation products) with TBA under acid conditions and at elevated temperatures. The resulting MDA-TBA adduct can be measured directly by spectrophotometry at 532 nm or, more sensitively, by fluorometric measurement with excitation at 515 and emission at 553nm.

TBA assays are therefore a total measure of malondialdehyde itself together with aldehydes, hydroperoxides and cyclic endoperoxides (Gutteridge and Tickner 1978). This is the main reason that the level of lipid peroxidation is usually expressed as TBARS (thiobarbituric acid reactive substances).

The TBA assay is, however, subject to several disadvantages. TBA reacts with MDA as well as with other non-lipid compounds to produce non-specific compounds that lie within the same spectra (Sjodin *et al* 1990) and MDA levels are overestimated through the fluorimetric measurement of the MDA-TBA adduct (Vina *et al* 1998). It has also been suggested that there is only a small quantity of free MDA in biological samples and the majority of MDA measured is formed by the decomposition of lipid peroxides (LOOH) and further peroxidation during the heating stage of the assay (Holley and Cheeseman 1993).

In order to overcome some of these disadvantages, modifications have been suggested such as HPLC separation and fluorometric detection. Using such methods, the MDA-TBA adduct can be identified and separated from other TBA reacting substances. However, the suitability of a MDA analysis depends on the purpose of the investigation and the sample of interest. As an index of lipid peroxidation the TBA assay can perform well especially for relatively simple samples like urine (Hageman *et al* 1992). TBARS have been shown to increase in muscles and liver of exercised animals (Witt *et al* 1992). In humans, measurements of lipid peroxidation due to exercise are not consistent. Viinikka *et al* (1984) reported no increase in plasma lipid peroxidation with exercise in highly trained subjects but Sumida *et al* (1989) using a similar exercise

protocol but less well trained subjects found a slight but significant elevation in serum TBARS. Maughan *et al* (1989) found increased serum TBARS in men following a 45-minute downhill run. Plasma TBARS in horses were seen to increase following a 140 km endurance race, immediately after exercise and also at 16 hours into recovery, compared with pre-exercise values (Marlin *et al* 2002). Plasma TBARS measured in horses immediately after intense exercise over jumps was not significantly different to pre exercise values but was seen to significantly decrease after 24 hours of rest (Balogh *et al* 2001).

Generally, TBARS are measured in plasma or serum, however, breakdown products of lipid peroxidation are generally removed from the body into urine, or into inhaled air. Monitoring urinary excretion of breakdown products of peroxidised lipids, therefore, is a promising way of establishing oxidative damage (de Zwart 1998).

1.12.5 N-acetyl-β-D-glucosaminidase (NAG)

The enzyme N-acetyl-β-D-glucosaminidase (NAG) is an acid-lysosomal hydrolase enzyme with a molecular weight of between 130 000 and 140 000 daltons (Bickerstaff 1987). Lysosomes are cell organelles which release enzymes such as hydrolases, ribonucleases and proteases in an autophagic response to cellular damage. In the kidney, NAG is located predominantly in the proximal renal tubules (Taylor *et al* 1971) and its primary function as a lysosomal hydrolase is the catabolism of damaged cell components. NAG activity in the kidney provides a reflection of the level of renal insult, since any kidney cell insult results in NAG being synthesised and released. NAG

enzymuria has proved to be a useful indicator of renal insult preceding renal dysfunction in a variety of clinical disorders, including glomerulonephritis (Soejima and Nagasawa, 1983), hepatic disease (Kim *et al* 1983), diabetes mellitus and diabetic nephropathy (Whiting *et al* 1979).

During exercise, blood flow is shunted away from organs and tissues (e.g. kidneys) to the working muscles. Renal vasoconstriction occurs during maximal exercise in ponies and blood flow to the kidney is only about 20 percent of that measured in the resting horse (Manohar 1991). Other studies of renal flow, glomerular filtration rate and urine flow during maximal and sub-maximal exercise have shown a decrease in renal blood flow and glomerular filtration rate, which was most severe during high intensity exercise, there being a 70% decrease in renal blood flow (Schott 1991). The alteration in renal function is short - lived, there being a return to pre-exercise values shortly after the cessation of exercise (Schott 1991). However, after prolonged exercise where there has been extensive fluid loss in the sweat, the reduction in renal blood flow and glomerular filtration rate may continue because acute renal failure is a common complication of exhaustion in endurance horses. At the cessation of exercise these regions then undergo re-oxygenation and this may lead to a burst of ROS production that occurs during ischemia reperfusion injury (Witt *et al* 1992).

Post exercise urine samples were collected from 30 long distance runners, pre, immediately post and 6, 12, 24, 36 and 48 hours after exercise. NAG activity was significantly increased and did not return to pre-race values until 24-36 hours post exercise (Gilli *et al* 1984). Urinary NAG activities and total protein were also seen to

significantly increase in human subjects following a triathlon (Yaguchi 1998) and after a 100Km hill walk (Robertshaw *et al* 1993).

Total serum NAG was measured in diabetics and the values compared with oxidative stress as estimated by plasma MDA concentrations. There was significant positive correlation between serum NAG activities and plasma MDA concentrations supporting a suggestion that oxidative stress may influence serum NAG activities. There was also a decrease in NAG activities after antioxidant treatment with alpha tocopherol (Skrha and Hilgertova 1999).

In addition, a positive correlation has been observed between NAG enzymuria (Urinary NAG activity/creatinine) and age in human subjects (healthy, with no apparent kidney problems detected). The variability of the NAG index also tended to increase with ageing (Oba *et al* 1999). However, no correlation was observed between age and NAG activity in the urine of healthy cattle (Sato *et al* 1997).

1.13 BODY FLUIDS OBTAINED BY NON-INVASIVE METHODS

The traditional biological samples for qualitative and quantitative analysis are blood, plasma and urine. Many substances are present in different concentrations in these fluids. Blood or plasma provides an estimate of the concentration of the circulating analyte of interest. Urine permits the measurement of the accumulated concentration of analytes since the last void of the bladder. For the measurement of drugs, saliva was suggested as early as the 1970's as an alternative medium (Hold *et al* 1995). For many drugs the monitoring of saliva is a real alternative to determining plasma levels because

saliva lacks "the drama of blood, the sincerity of sweat and the emotional appeal of tears" (Mandel 1990).

1.13.1 Urine

Urine is a modified ultrafiltrate of plasma and is the most commonly tested body fluid for the detection and diagnosis of disease. It is the vehicle by which water and solutes in excess of body requirements are excreted, together with end products of metabolism. Most toxins are eliminated from the system by excretion in the urine and urine analysis can provide an accurate diagnosis of problems occurring within the urogenital tract.

The kidney

In general kidneys regulate the volume and composition of body fluids. They conserve fluid components necessary to maintain homeostasis while ridding the body of metabolic waste products as well as excess water and electrolytes in the form of urine. The kidneys of the horse are heart shaped and lie against the diaphragm and psoas muscles dorsally, each of them enclosed by a capsule of fat. The right kidney lies ventral to the last two ribs and the left kidney ventral to the last rib. Each equine kidney weighs approximately 700 grams.

The kidney consists of the cortex (the outer region) which is dark in colour and contains the filtering units, or nephrons. The medulla (the inner region) is lighter in colour than the cortex and contains the collecting ducts. The renal pelvis is a cavity in the centre of the kidney and urine collects here prior to leaving via the ureter. Kidneys of the horse

are a modified unipyramidal, or unilobar, where there is a single medullary mass that confines the cortex to the periphery where it forms a continuous shell (Dyce *et al* 2002). The absorption system of the kidney consists of the glomerulus, the proximal convoluted tubule, the loop of Henle, the distal convoluted tubule and the collecting duct, collectively, called the nephron. The substance of the kidney consists of several hundred thousand nephrons.

The glomerulus consists of a tuft of capillaries coiled into a cup-shaped funnel. The blood vessels are called the glomerulus and the funnel is known as Bowman's capsule. The glomerular membrane consists of three layers; the capillary wall, composed of endothelial cells, the basement membrane and the epithelial cells of Bowman's capsule. The epithelial cells possess "foot processes" which are interdigitating prolongations of the cell which are in contact with the basement membrane leaving fine spaces between the foot processes. There are charge barriers in the basement membrane which cause negatively charged molecules to be filtered less than neutral ones, but substances up to 14 Angstroms in size can pass freely and the size limit is assumed to occur around 36 Angstroms (Mcleod 1995).

Glomerular filtration is a passive process and molecules such as glucose, urea, amino acids, electrolytes, pass freely into glomerular filtrate. Plasma proteins are too large to pass through and are almost completely excluded. The glomerular filtration rate is affected by the pressure of blood in the glomerular capillaries, which in turn depends on the arterial blood pressure, the concentration of plasma and the intratubular hydrostatic pressure.

The glomerular filtrate is modified in the tubules by reabsorption of water and many solutes. The volume and sodium and chloride contents of the filtrate are reduced by approximately 80% in the proximal tubules, while virtually all the glucose, amino acids, and potassium are reabsorbed (Walker 1990).

The loops of Henle move salt ions between each limb to return water to body fluids. Of the descending and ascending limbs, only the former is permeable to water. By moving sodium into the tissue fluids from the ascending limb water is attracted out of the descending tubule, therefore returning water to the tissues. Active transport of sodium depends upon passive movement of chloride in the opposite direction and occurs mainly in the proximal tubules.

The distal convoluted tubule uses cation exchange to adjust concentrations of substances and water volume. In the distal nephron the action of antidiuretic hormone (ADH) ensures that water is reabsorbed resulting in reduced urinary volume. Water reabsorption continues in the collecting ducts until the filtrate is at the correct volume, composition and osmolality. At this point the substance is urine and drains into the renal pelvis to travel down the ureter to the bladder.

Within a 24 hour period the average horse will produce between 2 and 11 litres of urine but many factors will affect the amount of urine excreted including water intake, salt intake, exercise and digestibility of the diet (West 1988).

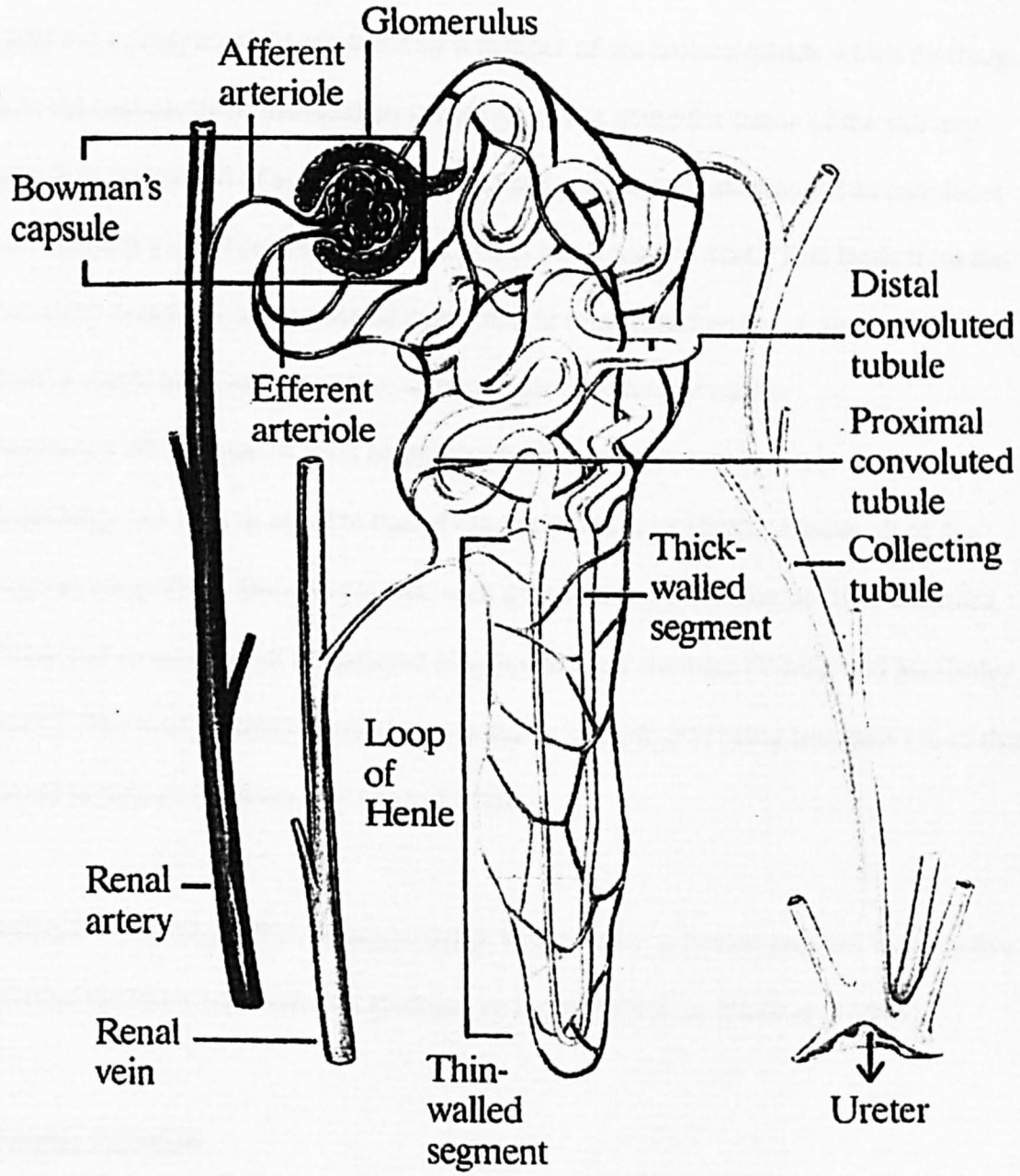


Figure 1.5 Structural Relationships of the Nephron (Walker 1990)

1.13.2 Saliva

Saliva is a complex fluid produced by a number of specialised glands which discharge into the oral cavity of mammalian vertebrates. The glandular tissue of the salivary glands is comprised of acinar cells, specialised groups of cells arranged as endpieces surrounding a small central lumen that opens into a narrow duct. This leads from the secretory endpieces to the striated ducts, that in turn drain into the secretory ducts to form a single main secretory duct which drains into the oral cavity.

Saliva is a dilute aqueous fluid containing both electrolytes and protein with an osmolality less than or equal to that of plasma (Hold *et al* 1995). Almost all of the organic compounds found in plasma, such as hormones, immunoglobulins, enzymes, DNA and viruses can all be detected in saliva in trace amounts (Vining and McGinley 1985). The total protein concentration in saliva is negligible being less than 1% of that found in plasma (Breimer and Danhof 1980).

Saliva is formed by different mechanisms: by a passive diffusion process, by an active process against a concentration gradient, or by ultrafiltration (Hold *et al* 1995).

Passive diffusion

Highly lipid-soluble materials such as cortisol may cross the capillary wall, basement membrane and acinar cell of the secretory endpiece. The same mechanism would allow them to pass through the cells lining the duct of the gland.

Active transport

The acinar cells forming the secretory endpiece of the salivary gland actively pump sodium ions from the blood into the lumen of the endpiece. The resulting osmotic pressure difference between the blood and the fluid in the endpiece causes water to flow from the blood, through the tight junctions between the acinar cells and into the lumen of the endpiece. The primary secretion is thought to be almost isotonic with plasma (Hold *et al* 1995). As this initial fluid moves down the ductal system of the salivary gland, an energy dependent transport process reabsorbs sodium and chloride. Potassium, lithium and bicarbonate ions are actively secreted into saliva. The ductal membranes are relatively impervious to water so the resulting saliva becomes increasingly hypotonic as it moves down the ductal system (Hold *et al* 1995). The cells lining the ducts have a limited capacity to pump the sodium out of saliva and this capacity does not increase proportionally as saliva flow rate increases. Therefore, as the saliva flow rate increases so does the sodium ion concentration. Potassium, chloride and bicarbonate ion concentrations also show a marked dependence upon flow rate (Hold *et al* 1995).

Ultrafiltration

Small polar molecules such as glycerol and sucrose enter into saliva (Hold *et al* 1995). This mechanism is restricted to compounds with a molecular weight of less than 300 Da

and even those with a MW of 150 Da are only filtered to a minimal extent (Hold *et al* 1995).

10 –12 l of saliva are secreted daily in the horse (Frape 1998). The fluid has no digestive enzyme activity and its function is primarily lubrication and buffering, the bicarbonate content of equine saliva is approximately 50mEq/l (Frape 1998). However, equine saliva is lower in sodium and buffering ions than ruminants and there is not as much urea recycling as in ruminants.

1.13.3 Sweat

Horses depend on sweating as the primary mechanism for thermoregulation. Body water losses in sweat, urine and from the lungs during a prolonged exercise can approach 10-12 l/h and total as much as 40 l (Meyer 1990).

The total body water of a 450-500 kg horse is around 300 l. Of this, 200 l would consist of intracellular fluid and 100l of extracellular fluid (McConaghy 1994). The major components of the ECF are plasma, interstitial fluid, and fluid present in the gastrointestinal tract. Sweat is derived from the interstitial fluid, with subsequent transfer of plasma and cellular fluid to the interstitial space to maintain interstitial fluid volume. Consequently, sweating during exercise, depletes both intracellular and extracellular fluid. In a horse of 450-500 kg, 8-14% of total body water would be lost in extended exercise (Meyer 1990).

Control of sweating during exercise occurs by activity of the autonomic nervous system and adrenaline (Kerr and Snow 1983). Horses produce alkaline sweat, hypertonic relative to plasma (Kerr and Snow 1983), while high intensity exercise in horses produces more dilute sweat than low intensity prolonged exercise. This may be due to increased adrenaline concentrations that result during high intensity exercise (McConaghy 1994). There is little change in sweat electrolyte concentration with prolonged sweating in response to submaximal exercise (McConaghy 1994).

1.14 FACTORS AFFECTING OXIDATIVE STRESS IN HORSES

1.14.1 Exercise

A link between physical exercise, elevated O_2 consumption and free radical production has been established (Kanter 1998). It has been estimated that for every 25 O_2 molecules reduced by normal respiration one free radical is produced (McCord 1979). The rate of oxygen consumption during exercise will greatly increase and O_2 flux in an active muscle may increase by up to 100 - fold (Sen 1995), consequently, increased metabolism can lead to increased free radical production. In addition, transient tissue hypoxia which may occur with high intensity anaerobic work can lead to an increase in hydrogen ions, which can react with superoxide anions to produce additional reactive oxygen species (Jenkins 1993). Tissue hypoxia can also lead to the freeing of transition metals such as Fe and Cu from their normal transporters. These free metals can further catalyse free radical reactions (Kanter 1998).

It has also been suggested that reperfusion of hypoxic muscle after a period of stressful activity can result in perfusion reperfusion injury (Kanter 1998). During exercise, blood flow is shunted away from many organs and tissues to the working muscles and part or all of these regions may experience hypoxia; in addition during exercise at or above maximal oxygen uptake fibres within the working muscles undergo hypoxia. At the cessation of exercise these regions then undergo re-oxygenation and this may lead to a burst of ROS production that occurs during ischemia reperfusion (Witt *et al* 1992). The intensity and duration of the exercise appears to influence the appearance of lipid peroxidation, in that an increase is more likely to be seen at VO_2 max. It seems likely that intense or exhaustive exercise in untrained subjects is more likely to produce oxidative damage (Witt *et al* 1992).

1.14.2 Ageing

In order to cope with the natural fluctuations in oxidative stress, animals are able to adapt by the upregulation of both prevention and repair mechanisms (Davies 1995). However, despite the adaptability and complexity of the cellular defence mechanisms, oxidative damage is potentially inescapable.

The free radical theory of ageing proposed by D. Harman in 1954 suggests that ageing is a consequence of cell damage as caused by free radicals and some age - associated disorders are believed to be associated with the time-dependent shift in the antioxidant/pro-oxidant balance in favour of oxidative stress (Meydani 1999).

There are many different theories relating free radical mechanisms implicated in the ageing process (Blumberg and Halpner 1999). These are briefly described below:

- Oxyradical-induced DNA cross-links could lead to somatic mutations and loss of essential enzyme expression, leading to a decline in oxidative phosphorylation, inefficient electron transport, and increased oxidant flux.

- Oxidation of sensitive sulphhydryl groups could cause cellular damage to mitotic and cytoplasmic microtubules.

- Macro-molecular cross-links of connective tissue could impede nutrient diffusion and impair tissue viability.

One of the best supported theories of free radical implication in the ageing process is the mitochondrial damage theory. Free radical damage in cells and tissues is particularly prevalent in the mitochondria where over 90% of the oxygen consumed by a mammal is utilised. ROS generated within the mitochondria may pose a threat by having an accumulative deleterious effect upon the components of the respiratory chain. For example, NADH dehydrogenase is inhibited by $O_2^{\bullet-}$ (Harman 1994).
Studies have shown that mitochondria from senescent animal tissues had a higher rate of leakage of superoxide than from young animals (Miquel and Fleming, 1986; Nohl

and Hegner, 1978). In addition, mitochondrial rates of $O_2^{\bullet-}$ and H_2O_2 formation have been found to increase with age (Sohal and Orr,1992).

It has also been demonstrated that oxidative lesions in human DNA isolates are age-related (Ashock and Rahid 1999). ROS generated during normal oxidative metabolism can interact with the genetic apparatus of a cell and alter the appropriate state of differentiation.

Dysdifferentiation is defined as a change in the properties of cells over time, due to the accumulation of damage. It results in the cell being unable to perform properly (Cutler 1984) and can lead to both ageing and neoplastic changes (Cutler, 1991). Such alterations may accumulate with time, transforming both cells and tissues.

Free radicals appear to play an important role in the initiation of many chronic diseases such as cancer, rheumatoid arthritis, cataracts and atherosclerosis (Blumberg and Halpner 1999). Pryor (1984) suggested that the ageing effect of free radicals may simply be linked to their role in the aetiology and development of chronic diseases that are the most life-limiting.

1.14.3 Obesity and oxidative stress

Obesity is where an abnormal amount of fat accumulates beyond body requirements and is one of the commonest forms of malnutrition seen in companion animals.

Body condition scores are used in horses to appraise the amount of fat present on an animal and it has been shown, in horses, that body condition scores correlate with body

fat percentage (Henneke *et al* 1983). Henneke *et al* (1983) devised a system where a score of 1 is extremely emaciated and 9 is extremely fat, based on visual appraisal and palpable fat cover at six areas of the horses body (behind shoulder, ribs at mid-barrel, crest of neck, withers, crease of back, at the tail head).

This system was further adapted by Carroll and Huntingdon (1988) with an 11 point scale from 0 to 5 (0 being emaciated, 5 being obese), with 0.5 gradations.

Obesity in horses is a serious welfare concern in the equine industry. Not surprisingly excess fat reduces the horses' ability to dissipate heat loads during exercise, extra weight increases biomechanical loads carried by the limbs, and obese horses are at greater risk of hyperlipidaemia if deprived of food. The abnormally high levels of lipids are subsequently metabolised in the liver, which can then cause liver damage.

Obesity in horses is associated with abnormal glucose homeostasis, as the over fat horse becomes relatively resistant to the action of insulin.

Fat ponies were found to be far more intolerant to oral glucose loading than normal ponies or Standardbred horses (Field and Jeffcott, 1989). These ponies also demonstrated a greater response in plasma insulin levels after glucose loading. Insulin response tests showed only a minimum and protracted response in the fat ponies, demonstrating insulin insensitivity in these animals.

This has important implications for the pathogenesis of laminitis and obese horses are indeed at high risk of developing this disease.

In humans, obesity is also associated with insulin resistance, and there is evidence to suggest that insulin resistance is linked to oxidative stress, for example, hydrogen

peroxide impairs insulin signalling and inhibits glucose transport (Keaney *et al* 2003). In addition it has been reported (Ceriello, 2000) that insulin itself promotes hydrogen peroxide generation in fat cells.

Keaney *et al* (2003) used urinary isoprostanes (8-epi-$PGF_{2\alpha}$) to evaluate oxidative stress in 2828 human subjects (from the Framingham heart study). They observed a strong association between body mass index and urinary isoprostane levels. They found that each 5 kg/m^2 was associated with a 9.9% increase in urinary creatinine indexed isoprostanes.

To check that this result was a reflection of obesity waist to hip ratios were substituted for BMI and again this was significantly associated with urinary isoprostane levels.

Mutlu-Turkoglu *et al* (2003) measured serum MDA and the susceptibility of VLDL + LDL to oxidation in 48 obese women and 25 non-obese women. They found that serum MDA levels were increased in obese women compared to non-obese women and that VLDL + LDL oxidation was increased in obese women and this also correlated with BMI. In addition, Ozata *et al* (2002) found increased levels of erythrocyte TBARS and decreased activities of erythrocyte Cu/Zn-SOD and glutathione peroxidase in obese adult men. They found a positive association between MDA and glucose and an inverse association between erythrocyte SOD and GPX activities and glucose, again supporting the view that disturbances in glucose and insulin metabolism may be related to altered oxidative status. The authors concluded that obesity leads to oxidative stress which in turn may contribute to obesity related diseases such as atherosclerosis.

Previous studies have also examined antioxidants in obese subjects. Kuno *et al* (1998) analysed beta-carotene and alpha-tocopherol levels in plasma and LDL of obese girls. They found that plasma beta-carotene and alpha-tocopherol levels were relatively lower in obese girls than in normal controls, and that both LDL beta- carotene and alpha-tocopherol were significantly lower in obese girls. Decsi *et al* (1997) found significantly lower plasma alpha-tocopherol and beta-carotene concentrations in obese boys than in control subjects.

Another reason for increased oxidative stress observed in obese subjects could also relate to a change in lipid composition. Fatty acid composition of plasma phospholipids, triglycerides and sterol esters was determined in 22 obese children and compared with data obtained from 25 healthy controls (Decsi *et al* 1996). Obese children exhibited significantly higher values of arachidonic acid and dihomo-gamma-linoleic acid in plasma phospholipids and sterol esters and significantly higher values for gamma-linolenic acid in plasma sterol esters.

This study demonstrated significantly higher levels of n-6 long chain polyunsaturated fatty acids in plasma lipids of obese children than in age-matched controls.

The peroxidizability index, (the susceptibility of lipids to oxidative stress) which is related to unsaturated fatty acid content is therefore increased in serum lipids of obese subjects.

Kuno *et al* (1998) found that LDL of obese girls contained more polyunsaturated fatty acids (PUFA) compared with normal controls. The peroxidizability index indicated that obese girls had significantly higher values than normal controls.

The link between obesity and oxidative stress is clear, even if the exact mechanism is not. As well as health risks associated with obesity, such as laminitis in the horse, the increasing number of obese horses in the equine population may well be experiencing increased free radical damage, which may in turn actually increase the propensity of these horses to succumb to obesity-related illness.

1.14.4 Intake of polyunsaturated fatty acids (PUFAS)

Diets for horses are commonly supplemented with fats, usually in the form of vegetable oils. The constituent oils of most seeds and cereal grains are rich in n-6 polyunsaturated fatty acids such as linoleic acid. In addition, many horses are supplemented with unsaturated fish oil n-3 fatty acids to reduce inflammatory reactions in joint disease. These PUFAs are highly susceptible to lipid peroxidation, particularly when intakes of antioxidants such as vitamin E, the major lipid peroxidation chain breaking antioxidant in membranes are low (Jenkinson *et al* 1999).

Human trials have shown that supplementing the diet with relatively high levels of n-3 PUFA increased plasma indices of oxidative stress which was prevented by increasing α tocopherol intake (Brown and Whale 1990). Jenkinson *et al* (1999) found that whole

blood oxidised glutathione and urinary TBARS increased in humans consuming a high PUFA diet (15% food energy).

Draper *et al* (1984) found increased MDA excretion in urine of rats treated with a high polyunsaturated fatty acid (PUFA) diet and a vitamin E deficient diet.

In the horse muscle TBARS have been shown to rise following supplementation with fresh corn and soya oil, despite the naturally high concentration of vitamin E and the addition of antioxidants during manufacture of the oil (Frape 1998). Increased uses of n-3 or n-6 oils in the horse should be accompanied by an increase in vitamin E supplementation (Frape 1998).

1.14.5 Iron supplements

Most feeds for horses contain fairly high levels of iron and a deficiency is only likely to occur as a result of a heavy parasite load. Despite this, iron containing supplements are widely used in the equine industry in the belief that PCV and Hb concentration can be increased, but these supplements are of little value and may actually be detrimental.

Fe^{2+} reacts with hydrogen peroxide in the Fenton reaction to generate the hydroxyl radical

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

Also, in the presence of transition metal ions such as Fe^{3+}, ascorbic acid (Sen 1995) and other antioxidants such as flavonoids (Sakihama *et al* 2002) may actually act as pro-oxidants.

It is suggested that the efficiency of iron absorption is decreased during periods of iron overload (McDonald *et al* 1995). However, this means that iron may be available to participate in Fenton-driven free radical generation in conjunction with the gut microflora (Lund *et al* 1999). A study investigating the effect of an oral supplement of ferrous sulphate on free radical generation in faeces found that faecal iron increased significantly during the period of supplementation and that the production of free radicals in faeces also significantly increased (Lund *et al* 1999).

Plasma and colonic lipid peroxides were seen to increase in rats with induced colitis consuming an iron supplement (3%/kg diet) and plasma vitamin E and C were also seen to decrease (Carrier *et al* 2001).

These results suggest that unabsorbed dietary iron may increase free radical production in the colon to a level that could cause free radical damage (Lund *et al* 1999). This could be particularly harmful for horses who rely on anaerobic microflora in the hind gut to digest fibre.

Wright *et al* (1999) investigated the effects of high iron and low vitamin E diets on lipid peroxidation in rats. Serum α tocopherol levels were lower in rats supplemented with iron and lipid peroxidation in liver was significantly increased by high iron diets after 3 and 10 weeks of treatment. Lipid peroxidation however was not altered in colonic mucosa.

Feeding iron supplements to horses, therefore, may increase the risk of free radical damage, either within the body as a whole or within the gut.

1.15 Rationale

It is clear that free radicals cause cellular damage and that free radical damage can occur under a range of circumstances. This thesis is concerned with free radical damage in senior horses and in horses performing exercise.

There is a real lack of information regarding nutrient requirements for senior horses and given that many older horses still perform exercise there is a need to establish if there is an increased requirement for antioxidant nutrients.

In addition, the published data regarding free radical production in horses are generally concerned with muscle and blood samples, so a non-invasive approach is also needed. The development of non-invasive techniques for the horse means that stressful methods of sampling such as blood samples and muscle biopsies are avoided and the principle of the 'three Rs' is upheld. Also, the non-invasive collection of body fluids means that these techniques may be more accessible and more easily applied practically.

This thesis concentrates on non-invasive sample collection, to assess oxidative stress in senior horses and in exercising horses.

Urine, saliva and sweat can provide useful alternatives to blood samples and these samples are collected in the following studies.

Choosing a marker of oxidative stress

Urinary 8 – hydroxyguanosine, F2-isoprostanes and TBARS have been previously used to assess free radical damage in humans. Isoprostanes in plasma and bronchoalveolar lavage fluid of horses has previously been assessed (Kirschvink *et al* 1999) and plasma TBARS have been measured in horses (Marlin *et al* 2002, Balogh, 2000, McMeniman and Hintz, 1992).

There is some question as to the usefulness of urinary 8-hydroxyguanosine (Collins 1999) and analysis of isoprostanes is complex and costly. Moreover, TBARS is a widely recognised marker of lipid peroxidation and is commonly used. Increasingly, urinary TBARS is being used as a marker of free radical damage in human studies (Siciarz *et al*, 2001, Mikami *et al*, 2000, Jenkinson *et al*, 1999). It seems sensible, therefore, if 'novel' samples are to be used (i.e. saliva, sweat, urine) that a common technique is employed to assess free radical damage. So, urinary TBARS was used as the principle marker of oxidative stress, in these studies.

However, if urine is to be used as an alternative to blood, it would be useful to try to establish other novel markers of free radical damage as the choice is rather limited. Urinary NAG activity is usually used as an indicator of early renal insult. However, Skrha and Hilgertova (1999) found a positive correlation between serum NAG activities and plasma MDA. This suggests that NAG may be useful as another marker of oxidative stress and is easily measured in the urine of humans, rats, pigs, dogs and cattle. However, urinary NAG activity has never been measured in the horse. Therefore, urinary NAG was measured alongside urinary TBARS to assess the usefulness of this potentially new application.

In addition to simply measuring markers of free radical damage it is useful to have some indication of the antioxidant status of the animal as well. This is usually assessed using blood samples. Saliva was used in the following studies as an alternative to assessing antioxidant capacity of blood.

This thesis, therefore aims to address the following questions:

1. Can TBARS be measured in equine urine?
2. How useful are urinary TBARS as a measure of oxidative damage?
3. How can saliva be efficiently collected from an exercising horse?
4. Can saliva be used to assess antioxidant status?
5. Can urinary NAG activity be measured in the horse and does it change with age/exercise?
6. Do markers of oxidative stress change with age?
7. Can other age-related changes be detected in urine?
8. Are markers of oxidative stress affected by exercise?
9. Are these markers affected when exercising horses are supplemented with an antioxidant?

In addition, the composition of equine urine was quantified using ^{1}H NMR spectroscopy to detect any changes with respect to both age and gender.

CHAPTER 2

PRELIMINARY EXPERIMENTAL WORK

2.1 INTRODUCTION

The aim of this preliminary series of experiments was to determine suitable methods that could be further used for the non-invasive quantitative measurement of oxidative stress in horses.

If urine is to be used extensively as an analytical matrix then it is important to ensure that there is nothing unusual in the composition of equine urine and that any differences in the urine composition of males and females is known and characterised. The composition of urine depends on many factors including diet, water intake, electrolyte intake, exercise, renal function and stage of oestrous cycle in the mare. Kinslow *et al* (1995) found that sudden increases in the urinary excretion of Na^+, Ca^{2+} and Mg^{2+} (mmol/24 hours) were seen in urine collected during the period from 2 days preceding oestrus to 4 days following. It was suggested that ovarian steroid hormones may exert an influence on urinary excretion of electrolytes in the mare.

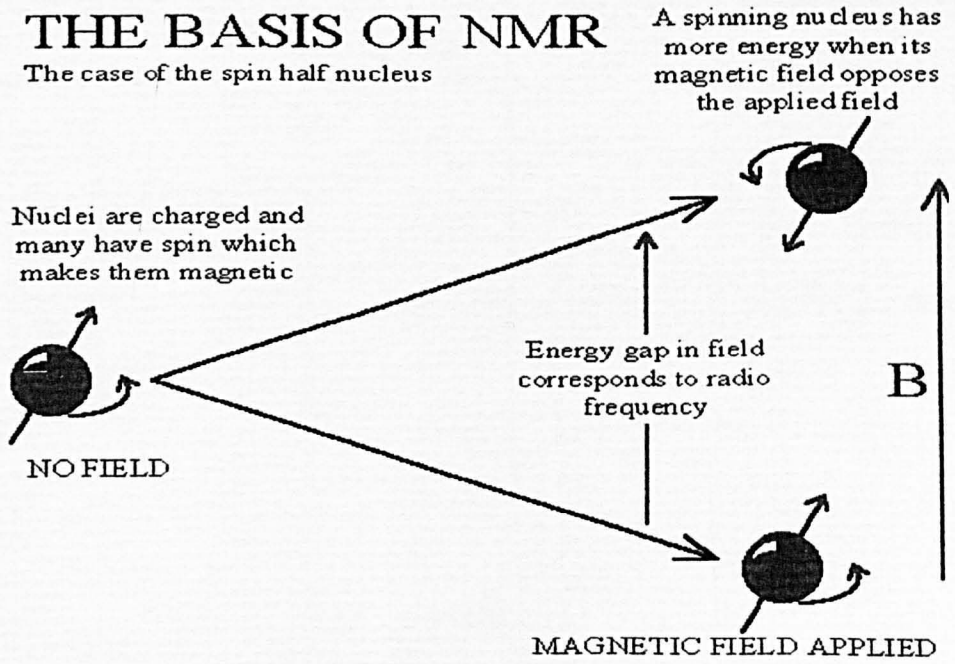
2.1.1 Nuclear Magnetic Resonance (NMR)

The nuclei of all elements carry a charge. When the spins of the protons and neutrons comprising these nuclei are not paired the overall spin of the charged nucleus generates a magnetic dipole along the spin axis and the size of this dipole is a fundamental nuclear property called the nuclear magnetic moment μ.

The symmetry of this charge distribution in the nucleus is a function of its internal structure and if this is spherical it is said to have a corresponding spin angular momentum number of I = ½ e.g. ^{1}H, ^{13}C, ^{15}N, ^{31}P.

For a nucleus with a spin of ½ there are two orientations of the nucleus when placed in a magnetic field; parallel to the field (low energy) and against the field (high energy). The parallel, low energy, orientation is more populated than the anti-parallel, high energy, orientation.

If the oriented nuclei are irradiated with electromagnetic radiation of the proper frequency the low energy state will absorb a quantum of energy and spin flip to the high energy state. When this spin transition occurs, the nuclei are said to be in resonance with the applied radiation. The amount of electromagnetic radiation necessary for resonance depends on the strength of the external magnetic field and on the characteristics of the nucleus being examined.



(http://members.aol.com/logan20/shift.html/)

The electrons in the molecule also have small magnetic fields associated with them and these oppose the applied field, screening the nuclei from the full strength of the applied field. The greater the electron density the greater this shielding will be, so nuclei in electron rich environments will undergo transition at a higher applied field than nuclei in electron poor environments.

The resulting shift in the NMR signal for a given nuclei is referred to as the chemical shift and in general protons adjacent to electronegative atoms will be deshielded and moved to a higher chemical shift. The following equation therefore applies:

Chemical shift (δ) = (shift observed/oscillator frequency) x 10^6 = ppm.

The precise resonant frequency is dependent on the effective magnetic field at the nucleus that is affected by electron shielding, which is, in turn, dependent on the chemical environment. The frequency dependence on the chemistry is what makes NMR such a powerful analytical tool. In general, the more electropositive the nucleus, the larger its chemical shift.

In proton NMR the intensity of the absorbance of a given class of nuclei is proportional to the number of protons giving rise to the signal – so the area under a given peak is directly proportional to the number of that type of proton present in the molecule.

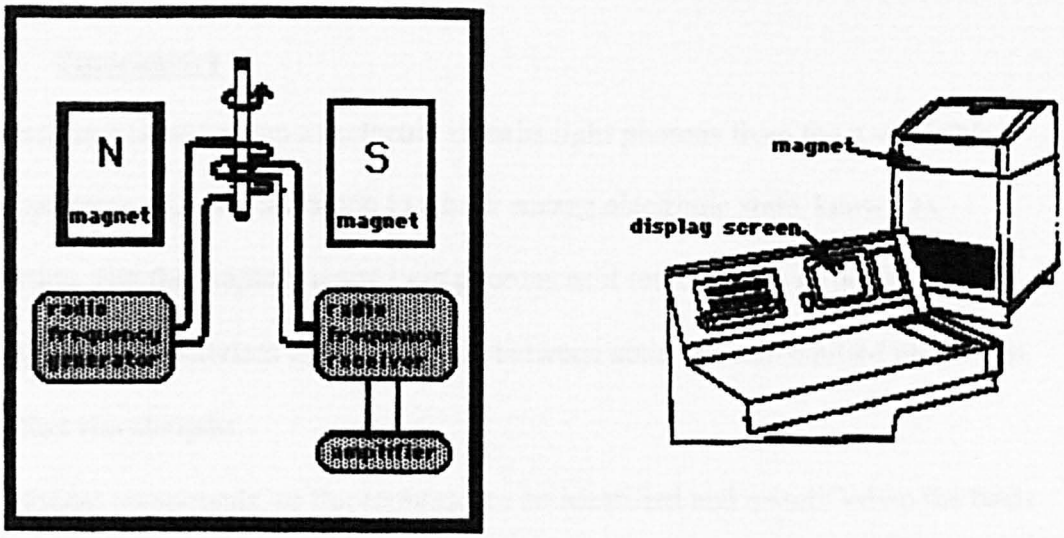


Figure 2.1 Components of a NMR spectrometer

(http://morgan.rutgers.edu/HTMLdocs/physics_modules/Magnetic_Resonance)

NMR can be used in a quantitative manner and to determine the characteristics of complex mixtures like urine, with little pre-treatment. The technique is rapid, requires only a small sample and involves no separation and derivatisation steps. An advantage is that ^{1}H NMR signals for creatinine provide a standard. Creatine phosphate is present in muscles and helps to provide energy for muscle contraction. Approximately 2% of creatine phosphate is converted to creatinine each day and is related to muscle mass (Varley *et al* 1980). As muscle mass remains relatively constant the amount of creatinine excreted on a daily basis remains the same. The integrated peak areas for many metabolites can be compared directly with those for creatinine and the concentrations expressed in terms of creatinine (Bales *et al* 1984). The aim of this study was to use proton – NMR spectroscopy to characterise equine urine and to determine any differences between males and females.

2.1.2 Fluorimetry

Fluorescence occurs when a molecule absorbs light photons from the u.v.-visible light spectrum, causing transition to a high energy electronic state, known as excitation, and then rapidly emits light photons as it returns to its initial state. Fluorimetry characterises the relationship between absorbed and emitted photons at specified wavelengths.

Fluorescent compounds, or fluorophors can be identified and quantified on the basis of their excitation and emission properties. Maximum emission occurs only for specific excitation and emission wavelength pairs, but the magnitude of fluorescent intensity is dependent on the intrinsic properties of the compound, the intensity of the absorbed light and the concentration of the fluorophor in solution.

An advantage of fluorescence over absorption spectroscopy is the ability to separate compounds on the basis of either their excitation or emission spectra, as opposed to a single spectra. A second advantage is low signal to noise since emitted light is read at right angles to the exciting light. For absorption spectrophotometry, the excitation source, sample and transmitted light are configured in line so that the absorption signal is the small difference between the exciting light and the transmitted light, both of which are quite intense.

The aim of this preliminary experiment was to establish if a fluorimetric method adapted from Yagi (1976) could be used to measure TBARS in the urine of horses.

2.1.3 Flavonoids

Flavonoids are phenolic compounds of vegetable origin with antioxidant effects. They vary in structure forming six classes: flavanones, flavones, flavonols, isoflavonoids, anthocyanins and flavans. They can scavenge free radical species such as peroxyl and hydroxyl radicals as well as ROS such as hydrogen peroxide. They act as antioxidants by donating electrons and phenoxyl radicals are formed as the primary oxidised product. These are rapidly enzymatically recycled to parent phenolics.

The antioxidant activities of a flavonoid depend on the number of hydroxyl substitutions in its backbone structure. In general, the more hydroxyl substitutions the stronger the antioxidant activity. Flavonoids that contain multiple hydroxyl substitutions showed antiperoxyl radical activities several times stronger than Trolox, a α tocopherol analogue (Cao *et al* 1997).

Flavonoids are thought to aid in the protection of other antioxidants such as vitamin E from oxidation. Viana *et al* (1996) investigated the *in vitro* effect of flavonoids on low density lipoprotein (LDL) oxidation and found that when flavonoids were present in the media vitamin E consumption was delayed in a concentration dependent manner.

Filipe *et al* (2001) used diluted human whole plasma to study the influence of flavonoids on lipid peroxidation promoted by copper, as assessed by TBARS and free MDA. They found that TBARS and free MDA formation increased during the incubation of plasma with copper and that addition of flavonoids inhibited copper induced lipid peroxidation.

Ishikawa *et al* (1997) measured the oxidisability of low-density lipoprotein (LDL) *in vitro* by measuring conjugated diene, TBARS and lipid peroxides after cupric sulphate was added. Flavonoids from tea (catechins and theaflavins) significantly and dose-dependently prolonged the lag time before initiation of oxidation. In the same study 14 healthy volunteers consumed 750ml black tea/d for 4 weeks. LDL oxidation in plasma was investigated in the same way and after consumption of tea the lag time before LDL oxidation was significantly prolonged, and no change was seen in 8 control volunteers.

Dandelion *(Taraxacum officinale)*

Dandelions contain flavonoids, terpenoids, inulin and high levels of vitamins and minerals. In particular, dandelion has been reported to contain the flavonoid luteolin (Williams *et al* 1996, Hu and Kitts 2003, Kvasnicka *et al* 2003). Fresh leaves are also reported to contain more β carotene than raw carrots and high levels of vitamin E (Leung 1985).

Milk thistle *(Silybum marianus)*

The active ingredient is silymarin, which has been shown to consist of a large number of flavonolignans including principally silybin accompanied by isosilybin, dehydrosilybin, silydianin and silychristin. Silymarin has been reported to have antioxidant properties (Flora *et al* 1998;Von Schonfeld *et al* 1997) and milk thistle has been shown to have clinical applications in the treatment of toxic hepatitis, cirrhosis, fatty liver and hepatitis (Luper 1998).

The aim of this experiment was to investigate the response of urinary TBARS to a dietary antioxidant supplement in the form of dandelion or milk thistle. These

particular antioxidants were chosen as herbs are frequently given to horses in the dried form, as supplements, but in addition these plants are commonly found in pastures and widely available to the horse.

Analysis of the herbs was also undertaken to establish that these supplements actually contained antioxidant flavonoids.

2.1.4 Free radical scavenging activity of saliva

All living cells possess a number of protective mechanisms against damage caused by free radicals and reactive oxygen species. Because components of saliva are derived from plasma it would be expected to have a free radical scavenging activity comparable to that of blood (Atsumi *et al* 1999).

Atsumi *et al* (1999) performed a trial to estimate the total free radical scavenging activity in the low-molecular-weight non-enzymic fraction of human whole saliva. Free radical scavenging activity of saliva and serum showed a significant linear relationship and physical exercise markedly decreased the activity. There was no difference between different ages and gender in their study.

The assay is based on the reduction rate of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Blois 1958). This has been frequently used to measure the antioxidative activity of natural foods such as tea catechin and flavonoid (Atsumi *et al* 1999). The decrease in the absorbance of DPPH at 517nm was measured after addition of a free radical scavenging agent, namely saliva.

The free radical scavenging activity of saliva gives an indication of antioxidant activity in saliva and therefore the assumption is made that it would also reflect the antioxidant activity in the plasma. On this basis it was decided to measure free

radical scavenging activity of whole equine saliva with a view to using this as a non-invasive marker of oxidative stress during exercise.

2.1.5 Urinary NAG activity

Urine NAG activity is a sensitive marker of the progression of renal disease and its activity increases before results of other renal function tests become abnormal (Sato *et al* 1997).

The natural substrate for NAG in the kidney is not known so an artificially synthesised substrate was used, namely, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-NAG). NAG splits the substrate to form 4-methylumbelliferone (4 MU) and N-acetyl -β-D-glucosamine. 4 MU is a fluorophor with excitation and emission wavelengths of 360 and 440 nm respectively at a pH > 10.4, however, it was anticipated that the signal would be sufficiently high to use a u.v./vis spectrophotometer. The assay used was adapted from that of Dance *et al* (1969).

The aim of these studies was to measure the activity of NAG in the urine of horses with a view to using NAG alongside TBARS to measure renal insult and oxidative stress during exercise and ageing, respectively.

2.2 MATERIALS AND METHODS

2.2.1 Use of NMR to characterise equine urine

Urine was collected from one mare (8 years old) and one gelding (15 years old) using Equisan urine and faeces collection harnesses (Arkenfield Racing Services, Nottingham).

Figure 2.2 Arthur modelling a gelding Equisan urine and faeces collection harness.

Multiple samples (n = 8) were collected from each horse, 4 samples were collected between 08.00 and 11.00 and 4 were collected between 15.00 and 18.00 hours. The samples were centrifuged at 3000rpm for 10 minutes to remove any sediment. The samples were then transferred to 1.5ml Eppendorf tubes and stored at –18°C. Duplicate samples of each of the urine samples were frozen using liquid nitrogen and placed overnight in a freeze dryer. 1ml heavy water (D_2O, 99.9%v/v), which also included an internal standard, was added. The treated urine samples were centrifuged at 10,000 rpm for 5 minutes. An aliquot (0.5 ml) of the supernatant was transferred to NMR tubes and analysed using a Bruker 400MHZ ^1H NMR instrument.

2.2.2 Measurement of urinary TBARS

Materials

All reagents were purchased from Sigma Aldrich (Poole, Dorset) and were of analytical grade.

Thoroughbred or Thoroughbred type horses were used (n = 14, 6 mares and 8 geldings). The majority of the horses (n = 9) were working in the school, or were being hacked out, between 1 and 2 hours per day, and 5 horses were not working at all.

Urine samples were collected using Equisan urine and faeces collection harnesses (Arkenfield Racing Services, Nottingham), 2 samples per horse wherever possible. The samples were transferred to 1.5ml Eppendorf tubes and stored at –18°C.

All urine samples were centrifuged at 3000rpm prior to analysis to remove any sediment, were diluted 50 fold with distilled water and were analysed in duplicate.

The urine samples were analysed for TBARS using the method adapted from Yagi (1976) with a luminescence spectrometer (Perkin Elmer LS 30).

The TBA reagent was made up from equal volumes of thiobarbituric acid (0.67% w/v) and glacial acetic acid. TBA reagent (0.5ml) was added to 2ml of sample and incubated in a boiling water bath for 30 minutes. The samples were cooled in ice, and 2.5 mls of 1 – butanol were added. The solutions were shaken and the resulting butanol layer was read fluorimetrically using a Perkin Elmer LS 30 luminescence spectrometer, at emission wavelength 553 and excitation wavelength 515nm.

A range of standard solutions (0 – 80 pmol/l) were made up, using MDA stock solution malonaldehyde bis (dimethyl acetal) and treated in the same way to obtain a standard curve (fig 2.6).

In addition, all urine samples were tested with 'Multistix ™', pre and post exercise for those horses that were working, to detect any alterations in urine composition.

2.2.3 Analysis of dandelion and milk thistle

A liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method was used to isolate and assess the concentrations of antioxidant flavonoids in dandelion and milk thistle.

Preparation of sample

Two grams of dried herb were extracted 3 times with 20 ml methanol by sonication at room temperature for 30 minutes. The extracts were combined and centrifuged at 3000 rpm for 10 minutes to remove any solid material.

Standards of the flavonoids apigenin, luteolin and silymarin (Sigma Aldrich, Poole, Dorset) were prepared in methanol. The concentrations used were 104, 208, 417, 833, 1667 ng/ml.

Analyses were performed using a Agilent Technologies pump (Hewlett Packard 1100 series) with a CTC Analytics autosampler (CTC PAL) and a PE Sciex mass spectrometer (API 3000/PE Sciex).

Chromatography was carried out using a reverse phase analytical column with a mobile phase fast gradient consisting of acetonitrile and 0.1% formic acid. The flow rate was 0.8 ml/ minute and run time 5 minutes. The analytes were ionised using the TurbolonSpray ^{TM} interface in negative ionisation mode.
Detection was via tandem mass spectrometry (MS/MS) in the multiple reaction monitoring (MRM) mode.

LC-MS/MS conditions

Column, Phenomenex C18 Luna, 50 x 2.0 mm i.d (5 μm).

Mobile phase A = 0.1% Formic acid (aq)

Mobile phase B = 0.1% Formic acid in Acetonitrile

0 min, 5:95 (A:B), 3.8 min, 95:5 (A:B), 5.0 min, 5:95 (A:B). Flow rate 800 μl /min (split ca 200 μl into Sciex).

MS analyses were carried out in negative ionisation mode (-50 ev apigenin, -52 ev luteolin, -40 ev silymarin).

In order to minimise possible carry-over effects, methanol injections were run between samples where low concentrations followed high concentrations.

All data collection, processing (peak area integration and quantification) and storage were performed using the Analyst ™ software associated with the mass spectrometer.

Analysis

A single calibration line, containing each analyte, was prepared using spiked methanol solutions and injected onto the LC-MS/MS system. The plant extracts were then injected and the concentrations back calibrated.

2.2.4 Response of urinary TBARS to antioxidant supplementation

Thoroughbred horses of equal fitness and body condition, performing similar work (3 hours per day in the school) were used (n = 12, 8 geldings and 4 mares, mean weight 519 kg, mean age 15 years). They were stabled full time and maintained on similar diets of haylage and concentrates, with no additional supplements or additives.

The horses were divided into 2 groups (n = 6, 4 geldings and 2 mares). Urine samples were collected from each horse using Equisan urine and faeces collection harnesses, over the course of week 1. Following this, horses 1-3 from group 1 received 30g of dried dandelion leaves (Hilton Herbs) and horses 1-3 from group 2 received 30g of milk thistle (Dodson and Horrell) as an additive in their feed. The supplements were fed for 2 weeks and according to manufacturer guidelines.

During the course of the second week of supplementation urine samples were collected from all horses using the Equisan harnesses. The diets were then crossed over with horses 4-6 from group 1 receiving 30g dandelion and horses 4-6 from group 2 receiving 30g milk thistle. Again the supplements were fed for two weeks with urine collection occurring in the second week of supplementation. The urine samples were frozen at –18°C prior to analysis.

	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Group 1	Horses 1 – 6 basal diet **Urine collected**	Horses 1 – 3 30g dandelion Horses 4 – 6 basal diet	Horses 1 – 3 30g dandelion Horses 4 – 6 basal diet **Urine collected**	Horses 4 – 6 30g dandelion Horses 1 – 3 basal diet	Horses 4 – 6 30g dandelion Horses 1 – 3 basal diet **Urine collected**
Group 2	Horses 1 – 6 basal diet **Urine collected**	Horses 1 – 3 30g milk thistle Horses 4 – 6 basal diet	Horses 1 – 3 30g milk thistle Horses 4 – 6 basal diet **Urine collected**	Horses 4 – 6 30g milk thistle Horses 1 – 3 basal diet	Horses 4 – 6 30g milk thistle Horses 1 – 3 basal diet **Urine collected**

Analysis

The samples were centrifuged at 3000rpm for 10 minutes to remove sediment. The method adapted from Yagi (1976) was used to determine TBARS, using a Perkin Elmer LS 30 luminescence spectrometer at emission and excitation wavelength of 553nm and 515 nm respectively (refer to section 2.2.2). Urinary creatinine was determined by the Jaffe reaction (Bonsnes and Taussky 1945), where a red colour is produced with an alkaline picrate solution. Urine samples were diluted 100 fold,

1ml of picric acid (40mmol/l) was added to 3ml of diluted urine, followed by 1ml of sodium hydroxide (750mmol/l). The samples were allowed to stand for 15 minutes and read within 30 minutes using a u.v./vis spectrophotometer (Jenway 6105) at 550nm. A blank consisting of 3ml water and 1ml each of the reagents was treated in the same way. Creatinine standards in the range 0 – 20 mmol/l were made up and analysed in the same way.

2.2.5 Measurement of free radical scavenging activity of equine saliva

2.2.5 (i) Collection of saliva

Two horses (geldings) were exercised on a high- speed treadmill for 27 minutes. They warmed up for 6 minutes at 2 m/s and 4m/s, exercised for 12 minutes at 2,4,6 and 8m/s and cooled down for 9 minutes at 4m/s and 2m/s. They were fitted with a bit specially designed to collect saliva for this study.

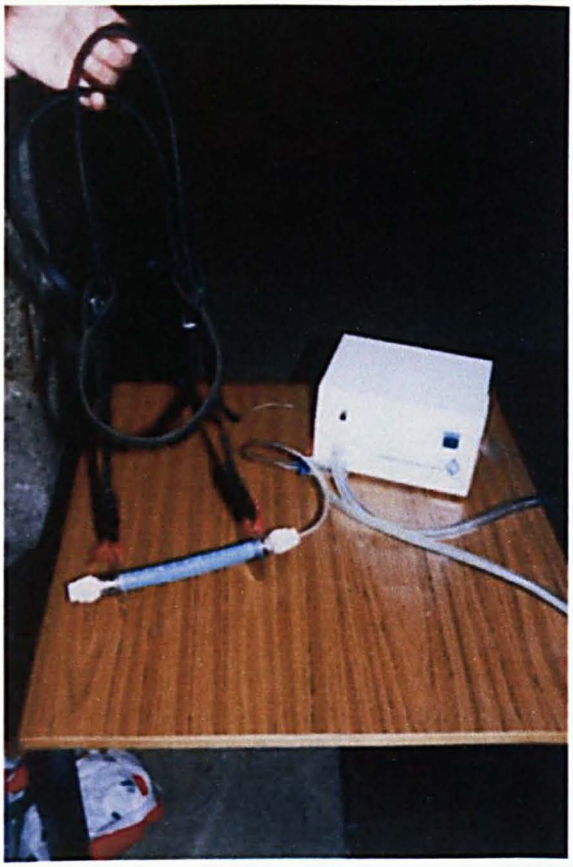


Figure 2.3 Saliva collecting bit

The bit consisted of an outer tube (medium density polyethylene 20mm outer diameter, 16mm inner diameter, pressure, 10 bar) and inner tube (15 mm outer diameter, 11mm inner diameter, pressure, 10 bar) with holes drilled through, with stoppers at each end (demountable push fit fittings). One end of the bit was connected to a silicone rubber tube, which was attached to a vacuum pump with a saliva - collecting trap in the middle. The vacuum pump was switched on after the horses had warmed up for 6 minutes and saliva was collected in the trap for the remaining 21 minutes.

Figure 2.4 Saliva collection using the specially designed bit. Saliva produced in the horses mouth runs into holes drilled along the length of the bit and is removed by gentle suction into the saliva trap illustrated.

2.2.5 (ii) Analysis

All reagents were purchased from Sigma Aldrich (Poole, Dorset) and were of analytical grade. The saliva samples were centrifuged at 10,000 rpm for 20 minutes and were analysed in duplicate using the method described below.

A solution containing 0.1mM 1,1 – diphenyl-2-picrylhydrazyl (DPPH), 0.9% NaCl and 25mM HEPES buffer, in 40% ethanol was prepared.

Saliva (0.1ml) was added to 0.9 ml of the DPPH solution and 1ml of DPPH solution was used as a control. The samples and control stood in the dark for 10 minutes and

were then centrifuged at 3000rpm for 10 minutes. The absorbance of the supernatant was measured in a u.v./vis spectrophotometer (Jenway 6105) at 517 nm. The free radical scavenging activity was calculated as

$$\frac{(A517\ control - A517\ sample)}{A517\ control.}$$

2.2.6 Measurement of N-acetyl-B-D glucosaminidase (NAG) activity in equine urine

All reagents were purchased from Sigma Aldrich (Poole, Dorset) unless otherwise stated and all samples were analysed in duplicate.

Urine was collected from 4 geldings of a similar age, post exercise, using Equisan urine and faeces collection harnesses (Arkenfield Racing Services, Nottingham). The samples were centrifuged for 10 minutes at 3000rpm, to remove any debris. Tubes were set up as follows in duplicate:

Reagent	**Test**	**Blank**
20mM trisodium citrate/phosphate buffer, pH 4.3 (with H_3PO_4)	0.9 ml	0.9ml
Sample (neat urine)	0.1ml	0.1ml
0.5 molar glycine buffer, pH 10.4 (with 5M sodium hydroxide)	-	3.0ml

The samples were allowed to equilibrate at 37°C for 10 minutes, then 1.0 ml of 2mM 4methylumbelliferyl-N-acetyl-β-D-glucosaminide (substrate) was added. The samples were incubated for 5, 10, 15, 20, 25, 30, 60 and 120 minutes. At each time interval, the reaction was terminated by adding 3.0ml of glycine buffer. Absorbance was measured in a u.v./vis spectrophotometer (Jenway 6105) at 360nm.

A standard curve was also prepared using 4 methylumbelliferone in trisodium citrate buffer in the range 0 – 160 nmol/ml. 2ml glycine buffer was added (final volume 3ml) and absorbance was measured at 360nm. A typical standard curve is given in figure 2.9.

2.2.6 (i) Gel filtration

25 ml of 0.15molar sodium chloride was passed through a sephadex column (G25 medium) and allowed to drain completely. Neat urine (2.5 ml) was added and the eluent discarded. 4 ml of 0.15M sodium chloride was added and collected. This was then analysed as before for NAG activity, with an incubation time of 30 minutes. The sephadex column was regenerated with 25ml of 3mmol sodium azide.

2.2.6 (ii) NAG activity in equine kidney tissue

One horse kidney was obtained from a local abattoir. 1g healthy tissue from the renal cortex was homogenised in 10ml of 20mM trisodium citrate/phosphate buffer, pH 4.3. The homogenate was centrifuged at 2000rpm for 10 minutes and the supernatant was frozen at – 18°C for 24 hours. The supernatant was thawed and analysed as for urine. The samples were incubated for 5, 10, 15 and 20 minutes.

2.2.6 (iii) Microcon filters

Urine was collected from 20 horses of different ages (age range 7-38 years). The samples were centrifuged at 3000rpm for 10 minutes to remove any debris.

Urine samples were then concentrated using a Microcon YM-50 centrifugal filter unit (cut off point of 50,000 daltons) (Millipore (UK) Ltd, Watford). Urine (0.5ml) was pipetted into the Microcon filter unit and spun at 13,000 rpm for 12 minutes. The concentrated sample was then analysed for NAG activity, with an incubation time of 15 minutes.

Urinary creatinine was determined by the Jaffe reaction (Bonsnes and Taussky 1945) using a Jenway 6105 u.v./vis spectrophotometer at wavelength 500nm (refer to section 2.2.3).

2.3 RESULTS

2.3.1 NMR

Figure 2.5 Example of a NMR trace of equine urine.

Key:
1 creatinine
2 Trimethylamine N-oxide (TMAO)
3 glycine
4 lactate
5 aromatic amino acids group 2 (phenylalanine and tyrosine)
6 aromatic amino acids group 1 (tryptophan)

Aromatic Amino Acids

The aromatic hydrogen atoms on phenylalanine resonate in the range 7.3-7.4 ppm and there are different peaks for the ortho, meta and para hydrogen atoms. There are 2 unique proton chemical shifts on the tyrosine ring near 7 ppm and tryptophan resonates at 7.1-7.2 and also at 7.6-7.8 ppm (http://www.isat.jmu.edu/users/klevicca/isat454/NMRChemscape.doc).

The peak referred to as group 1 occurs around 7.8 so this relates to the aromatic amino acid tryptophan. The substances resonating at 7 – 7.5ppm relate to phenylalanine and tyrosine, referred to as group 2.

PPM
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
1
2
3
4
5
6

Table 2.1 mean values from mare and gelding NMR spectra displaying standard deviations in parenthesis. All values are standardised against creatinine.

Sex	Aromatic amino acids group 1/ creatinine	Aromatic amino acids group 2/ creatinine	lactate / creatinine	glycine / creatinine	trimethyl AmineNoxide /creatinine
Mare	**1.05 (0.13)**	**3.7 (0.57)**	**0.74 (0.44)**	**0.94 (0.51)**	**0.25 (0.22)**
Gelding	**0.95 (0.17)**	**3.61 (0.48)**	**0.63 (0.06)**	**0.83 (0.25)**	**0.40 (0.12)**

The values were tested using a t score and no significant differences between the male and female samples or between samples collected in the morning and those collected in the afternoon were observed.

2.3.2 TBARS in equine urine

Table 2.2 TBARS in equine urine expressed as pmol/l MDA

Horse	sex	work	height/weight	MDA pmol/l
1	m	working	16.1hh 540kg	215
2	m	working	16.2hh 560kg	430
3	m	working	16.3hh 560kg	625
4	m	working	16.1hh 580kg	2375
5	m	working	16.3hh 560kg	718
6	m	working	16.2hh 580kg	1825
7	f	working	16.3hh 560kg	763
8	f	working	15.3hh 540kg	1515
9	f	working	16hh 530kg	1198
10	m	non - working	17.1hh 600kg	1275
11	m	non - working	16.2hh 560kg	1000
12	f	non - working	16.1hh 590kg	1350
13	f	non - working	15.3hh 540kg	2125
14	f	non - working	16hh 525kg	960

Mean = 1169.6 pmol/l

Standard error = 168.39

95% confidence interval for the mean = 805.8 – 1533.3 pmol/l

There was no significant difference between mares and geldings when compared using a student t test and also no significant difference between working and non working horses ($p<0.05$).

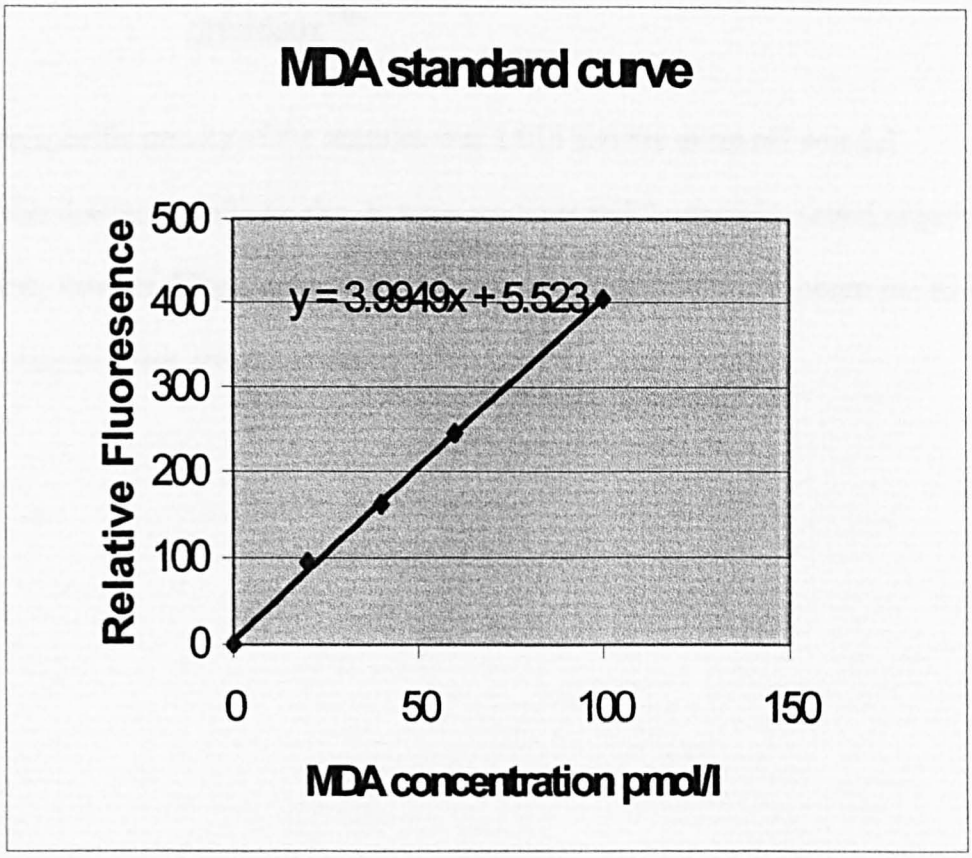


Figure 2.6 Typical MDA standard curve

2.3.2 (i) 'Multistix™'

The mean specific gravity of the samples was 1.014 and the mean pH was 8.1. Protein was detected in all samples, in trace amounts and the samples tested negative for glucose, ketones, blood, nitrates and leucocytes. No difference between pre and post exercise samples was observed.

2.3.3 Analysis of dandelion and milk thistle

The API 3000 LC-MS/MS TurbolonSpray™ interface gave optimum results in negative ion mode. The short, reversed phase column, with mobile phase at a flow rate of 0.8 ml/min, gave retention times of 2.27 for luteolin, 2.37 for silymarin and 2.45 for apigenin.

Apigenin, luteolin and silymarin gave quasi-molecular ions at m/z 269, 285 and 481 respectively. Fragmentation of these ions using CAD (collision activated dissociation) with a collision energy of ca –50 ev in the Q2 region of the mass spectrometer resulted in strong product ions, predominantly at m/z 117 for apigenin, 133 for luteolin and 125 for silymarin. Product ion spectra are given in appendix 1.

Apigenin, luteolin and silymarin were quantified in the plant extracts. Table 2.3 gives the concentrations of flavonoids identified.

Table 2.3 Concentration of flavonoids identified in dandelion and milk thistle. Values are presented for the extracts and per gram of herb (as fed).

Herb	Apigenin ng/ml extract	Apigenin mg/g herb	Luteolin ng/ml extract	Luteolin mg/g herb	Silymarin ng/ml extract	Silymarin mg/g herb
Dandelion	70	2.1	2500	75	/	/
Milk thistle	300	9	600	18	30	0.9

MRM chromatograms are presented in appendix 2

2.3.4 Response of urinary TBARS to antioxidant supplenentation

Group 1 – Dandelion supplementation

Table 2.4 Mean TBARS in equine urine, expressed as pmol MDA/mmol creatinine pre and post dandelion supplementation

Horse	Basal pmol MDA/mmol creatinine	Dandelion pmol MDA/mmol creatinine	No dandelion pmol MDA/mmol creatinine
1	709.4	234.3	33.0
2	118.4	97.5	37.5
3	399.6	139.1	31.3
4	184.8	/ *	343.6
5	490.1	57.0	83.9
6	326.2	22.6	316.3
Mean	**371.4 s.d. 195.8**	**110.1 s.d. 73.4**	**140.9 s.d. 135.1**

* no sample obtained

One way ANOVA ($P<0.05$) showed a significant difference between basal levels and treatment with additional dandelion and also a significant difference between basal levels and no additional dandelion treatment. There was no significant difference between the additional dandelion and no additional dandelion treatments.

Group 2 – Milk thistle supplementation

Table 2.5 Mean TBARS in equine urine, expressed as pmol MDA/mmol creatinine pre and post milk thistle supplementation

Horse	**Basal pmol MDA/mmol creatinine**	**Milk thistle Pmol MDA/mmol creatinine**	**No milk thistle Pmol MDA/mmol creatinine**
1	648.3	223.6	/ *
2	737.0	121.8	57.2
3	831.5	137.4	42.2
4	279.5	18.9	66.1
5	338.9	43.6	82.7
6	300.5	36.8	117.9
Mean	**522.6 s.d. 223.4**	**97.7 s.d.71.7**	**73.2 s.d.25.9**

* no sample obtained

One way ANOVA ($P<0.05$) showed a significant difference between basal levels and treatment with additional milk thistle and also a significant difference between basal levels and no additional milk thistle treatment. There was no significant difference between the additional milk thistle and no additional milk thistle treatments.

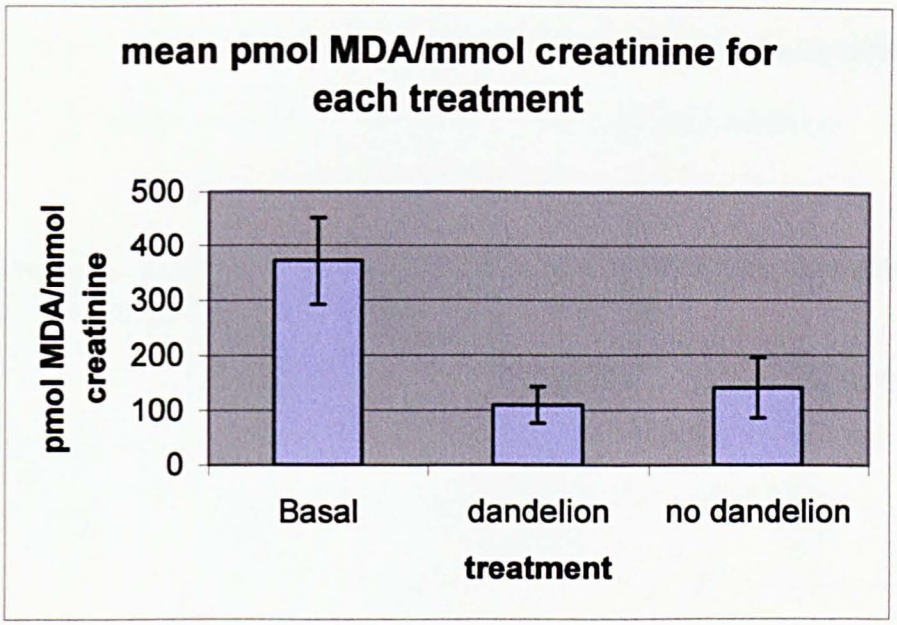


Figure 2.7 mean urinary TBARS pre and post dandelion supplementation showing standard errors of 79.9 (basal), 32.8 (dandelion), 55.1 (no dandelion)

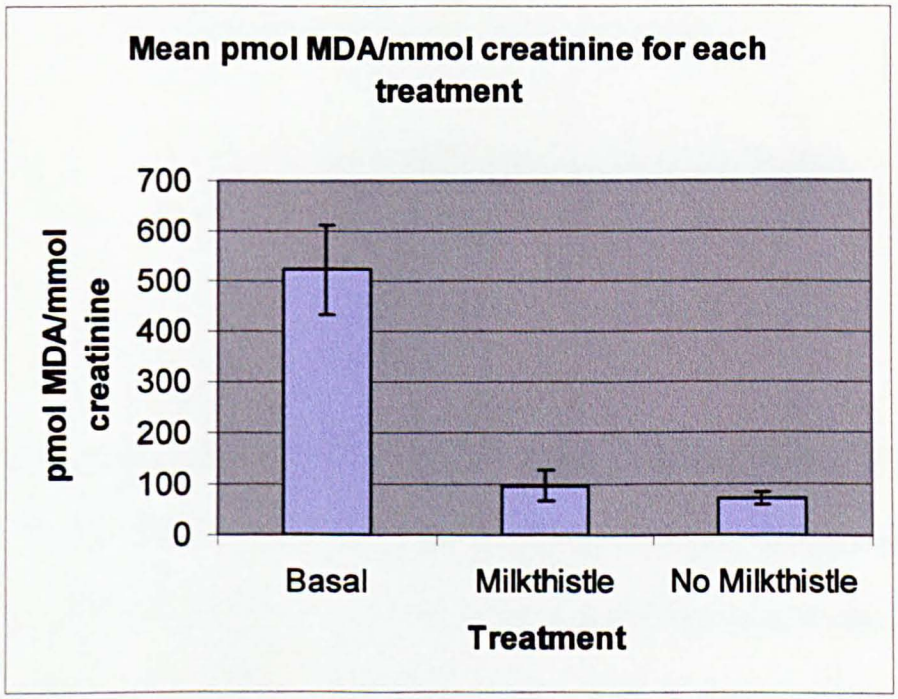


Figure 2.8 mean urinary TBARS pre and post milk thistle supplementation showing standard errors of 91.2 (basal), 29.2 (milk thistle) and 11.5 (no milk thistle)

The horses received 30 g of each herb daily as a supplement. The minimum amount of each antioxidant received by the horses in mg is given in table 2.6.

Table 2.6 Minimum amounts of flavonoids received by the horses on a daily basis from dandelion and milk thistle supplements (mg).

Herb	Apigenin	Luteolin	Silymarin
Dandelion	63	2250	/
Milk thistle	270	540	27

2.3.4 Free radical scavenging activity in saliva

Table 2.7 free radical scavenging activity of equine saliva

Sample	**μmol DPPH scavenged per ml of saliva**
Horse 1	0.260
Horse 2	0.345

The mean DPPH activities of saliva from 257 human subjects aged 4 – 72 years was 0.389 ± 0.190 μ mol/l (Atsumi *et al* 1999). These values were from the non-enzymic fraction of human saliva. The values obtained from equine saliva were comparable to these values, although the activity of whole equine saliva was measured in the present study.

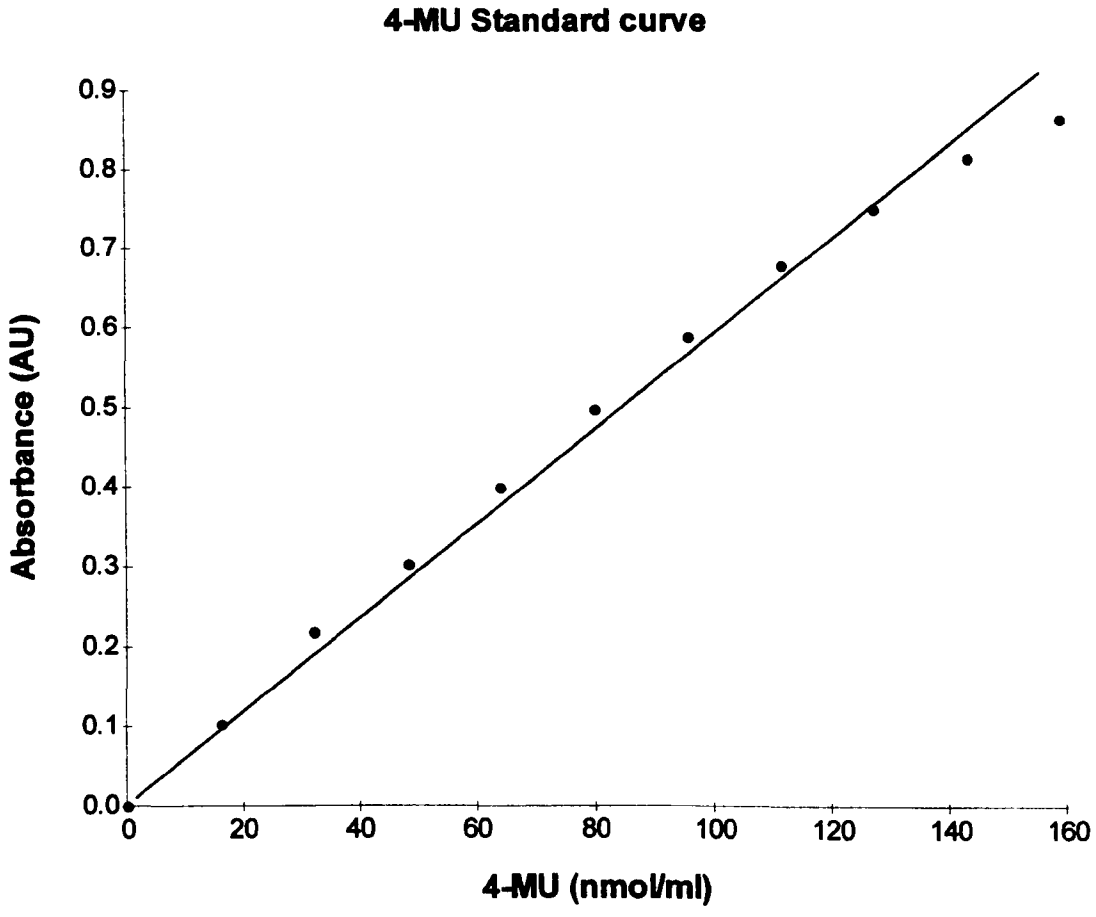
2.3.6 NAG activity in equine urine

Using the method published by Dance *et al* (1969) no NAG activity in horse urine was detected, using both spectrophotometric and fluorometric methods.

A standard curve was obtained (see figure2.9).

It is possible that there were low molecular weight inhibitors present in the urine samples, therefore, urine was next filtered through Sephadex columns in order to eliminate any potential inhibitors.

Figure 2.9 A typical 4-MU standard curve



2.3.6 (i) Gel filtration

Using the method published by Dance *et al* (1969) no NAG activity in horse urine was detected after filtering the urine through the Sephadex column.

NAG activity was therefore measured in renal tissue to determine whether or not NAG was present in the equine kidney.

2.3.6 (ii) NAG activity in equine kidney

Table 2.8 NAG activity in equine kidney tissue

Incubation Time	**Concentration of 4MU μmol/l**
(mins)	
5	41.58
10	64.58
15	121.67
20	153.67

μ mol 4MU formed per minute = 7.64

Enzyme activity, U/g wet tissue = 76.4

(U = 1μmol 4MU formation per minute).

NAG activity was detected in equine tissue at a value of 76.4 U/g wet tissue.

2.3.5 (iii) Microcon filters

Microcon filters have the dual functions of eliminating substances of 50,000 daltons and below, thus eliminating any potential inhibitors and also of concentrating the sample. Typical recovery rates from the Microcon filter were 0.1ml (from 0.5ml). NAG activity was detected in the urine of two horses out of twenty. The enzyme activities in urine from these two horses are presented in table 2.9

Table 2.9 NAG activity in equine urine

Horse i.d	4mu nmol/ml	U/L	Creatinine (g/l)	U/g creatinine
6	5.40	0.36	2.16	0.17
20	3.69	0.25	1.94	0.13

2.4 DISCUSSION

2.4.1 NMR

The Equisan urine and faeces collection harnesses were useful for urine collection as they could be left on the horses for a period of time (although they needed to be emptied on a regular basis) allowing the horse to urinate freely. Some horses however, were very inhibited by the harness and would go for long periods of time without urinating, so in these cases sometimes urine collection using a plastic bucket was more successful.

The gelding urine and faeces collection harnesses are designed so that urine and faeces are collected separately with no faecal contamination of the samples. In the mare, although the faeces and urine are collected in different parts of the harness, a small amount of faecal contamination sometimes occurs, but not to any great degree.

Equine urine is usually highly sedimented and although one of the advantages of using NMR is that the samples require little pre-treatment it is important to thoroughly remove this sediment from the urine as it interferes with the analysis. Also, if any samples contained residual water from the freeze drying process the water signal produced masked the other signals, so it is important to ensure that the samples are thoroughly freeze-dried.

There were no significant differences shown between mares and geldings with regard to the substances identified and quantified from the NMR data. The biggest difference was seen between the values for trimethylamine N-oxide (TMAO), with the gelding exhibiting a higher value. Methylamines, such as TMAO, are used to maintain intracellular levels of organic osmolytes. This occurs in renal medullary

cells which are subjected to an environment with a very high osmolarity. To balance high interstitial osmolarity, cells of the medullary structures must generate these organic osmolytes (Kaneko *et al* 1997). TMAO excretion will increase if these medullary cells are damaged. The gelding used in this study was 15 years old and the mare was 8 years old, suggesting that medullary integrity may decrease with age.

Although limited conclusions can be drawn from just 2 horses it seems likely that there are minimal differences between male and female equine urine but that there may be a difference in TMAO excretion with age.

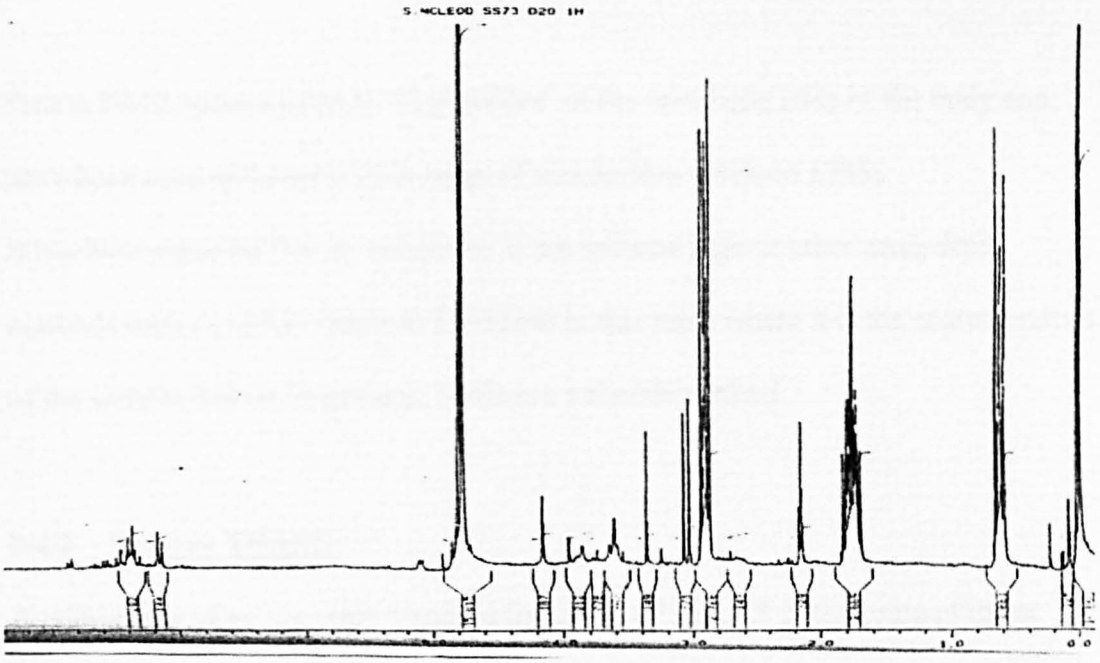
The concentrations of some of the major urine components in human urine are given in table 2.8 (adapted from Bales *et al* 1984).

Table 2.10 concentrations of metabolites in human urine as measured by NMR spectroscopy

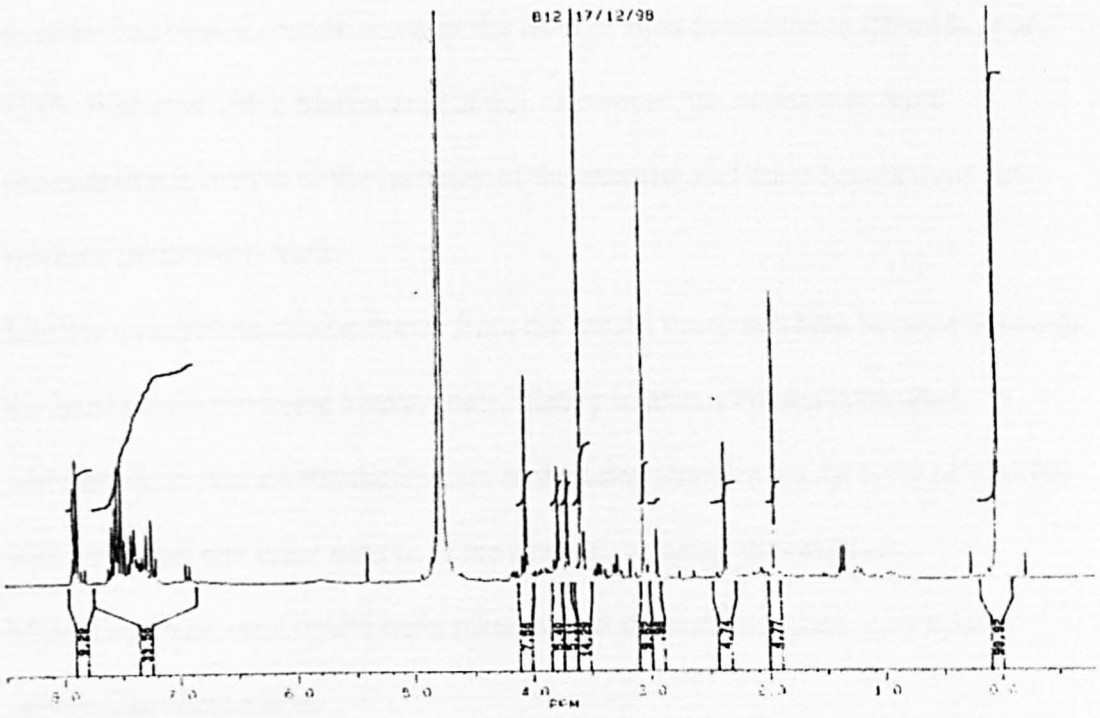
Compound	**ppm**	**Metabolite/creatinine**
Creatinine	3.50	/
Citrate	2.65	0.76
Glycine	3.57	1.02
Alanine	1.48	0.42
Lactate	1.34	0.38
Histidine	6.65	1.9

The value for lactate in human urine is lower than that obtained in equine urine, however the equine urine samples were mainly collected after exercise. Bales *et al* (1984) reported that the ^{1}H NMR spectrum of human urine collected after hard exercise showed a large increase in lactate content.

A difference between NMR traces of humans and horses is that a peak of allantoin can be seen at a chemical shift of 5.43 on the NMR trace of some of the horses This appears to be absent from the human NMR trace. Uric acid is the end product of purine degradation and in most animals the enzyme uricase converts uric acid to allantoin. Humans lack this enzyme, hence excrete higher levels of uric acid.



NMR spectra of human urine



NMR spectra of equine urine

Figure 2.10 Comparison of equine and human urine – note the peak of allantoin at 5.4

Proton NMR spectra provide 'fingerprints' of the metabolic state of the body and have been used to detect a wide range of metabolites (Mcleod 1995).

It has been reported that its sensitivity is not quite as high as other analytical methods such as HPLC (Mcleod 1995) but in this case, where it is the characteristics of the sample that are important, NMR is a valuable method.

2.4.2 Urinary TBARS

A wide range of values were obtained for levels of TBARS in the urine of these horses. There appeared to be no difference in the values for male and female horses and also no difference between working and non – working horses was noted. Higher levels of TBARS would be expected in the urine of working horses as exercise has been shown to increase the level of lipid peroxidation (Sumida *et al* 1989; Witt *et al* 1992; Marlin *et al* 2002). However, the increase in lipid peroxidation is related to the intensity of the exercise and these horses were not working particularly hard.

Limited conclusions can be drawn from the results presented here because although the horses were receiving similar diets, dietary intakes were not controlled. In addition, there was no standardisation of the urine samples, so the level of TBARS will vary from one urine sample to the next. Expressing the results as MDA/creatinine ratio would have standardised the results and allowed a more appropriate comparison.

It does appear that the heaviest horses have the higher levels of TBARS and this could suggest that the more muscle mass there is the greater the potential for free

radical damage. An interesting point is that horses number 4, 6, 12 and 13 were actually quite fat (condition score 4, on a scale 0, emaciated – 5, obese, Carroll and Huntingdon, 1988) and these horses have the highest levels of TBARS in the urine. A link between oxidative stress and obesity has been demonstrated in humans and it may be the case that either altered glucose metabolism or increased levels of polyunsaturated fats in obese horses may lead to an increase in urinary TBARS. There have been a number of criticisms aimed at the TBA assay (Sjodin *et al* 1990, Vina *et al* 1998, Holley and Cheeseman 1993). However, although the assay may be non-specific for any one class of peroxidation product it is a valid measure of overall peroxidative alterations.

Not many studies are known in which non - invasive *in vivo* samples were used but determination of MDA in urine has been used to establish the involvement of lipid peroxidation in humans (Siciarz *et al* 2001, [a]Mikami *et al* 2000, [b]Mikami *et al* 2000, Jenkinson *et al* 1999).

This study established that TBARS could be measured in equine urine using the method adapted from Yagi (1976), therefore, this assay can be used in further studies.

The 'Multistix ™' were used to detect any abnormalities in the urine, although these clinical sticks are designed for use in humans, not horses. No abnormalities were detected and there was no difference pre and post exercise.

All horses had trace amounts of protein in their urine. Rudolph and Corvalan (1992) found that 36% of urine samples from Thoroughbred horses showed traces of protein.

The level of proteinuria has been found to increase with exercise intensity (Schott *et al* 1995), but in this experiment the horses were not working particularly hard and there was no difference between pre and post exercise samples.

Wood *et al* (1990) found the pH of equine urine to be between 7 and 8.3. The mean value of 8.1 falls within this range. The addition of concentrates to the diet can cause a decrease in urinary pH (Wood *et al* 1990) but exercise is also an important factor. Horses performing endurance type exercise may have an increased urinary pH. Snow *et al* (1982) found a mean increase in urine pH from 7.4 to 8.4 in horses that were ridden over a distance of 80 Km. Stanley *et al* (1995) found that racing Thoroughbreds had decreased urinary pH, some as low as 5.5. However, the pH of the urine samples in this study did not change with exercise.

2.4.3 Analysis of dandelion and milk thistle

Previous studies have used HPLC with either u.v. or MS detection to analyse these flavonoids in plant material (Williams *et al* 1996, Hu and Kitts 2003). However, LC-MS/MS was used in the current study because of its high specificity.

The use of LC-MS/MS proved to be an excellent and novel method for detecting and quantifying flavonoids in plants. Monitoring product ions from quasi-molecular ions using multiple reaction monitoring gave excellent specificity and sensitivity. There were no peaks of any significance in the chromatograms that would compromise the method. Although autoinjector carry over was observed, it was at a level at which it did not compromise the quantification of the analytes. The levels of flavonoids in the plant extracts can only be considered as minimum values as recovery studies were not carried out. However, the method of extraction was an

adaptation of several methods used for extraction of flavonoids (Ku *et al* 2002, Pereira *et al* 2002) all of which gave excellent recovery.

Apigenin and luteolin were identified and quantified in dried dandelion leaf. The presence of luteolin in dandelion is consistent with other studies (Williams *et al* 1996, Hu and Kitts 2003, Kvasnicka *et al* 2003), but the presence of apigenin in dandelion leaf is not well documented. Hu and Kitts (2003) analysed solvent fractionated dandelion flower extracts using HPLC with u.v. detection and identified 25.20 mg/g luteolin in dandelion flower extracted into ethyl acetate. The current study detected 75mg/g luteolin in dandelion leaf.

High levels of luteolin were found in dandelion compared to the other flavonoids and there is evidence to suggest that luteolin is an effective antioxidant. Brown and Rice-Evans (1998) investigated the effect of a luteolin-rich artichoke extract on LDL oxidation *in vitro*. The artichoke extract retarded LDL oxidation in a dose-dependent manner and additionally, a 1mM solution of luteolin demonstrated a similar ability to inhibit lipid peroxidation *in vitro*.

Artichoke leaf extract also produced a concentration-dependent inhibition of oxidative stress in human leukocytes (Perez-Garcia *et al* 2000).

Apigenin is reported to be found in parsley (*Petroselinum crispum*) (Nielson *et al* 1999). In this study 14 human volunteers received a strictly controlled basic diet low in flavones which was subsequently supplemented with parsley providing 3.73-4.49 mg apigenin/MJ. Erythrocyte glutathione reductase and superoxide dismutase activities increased during intervention with parsley as compared with the levels on

the basic diet. Another study (Giles and Wei, 1997) investigated the effects of five selected flavones/isoflavones on the formation of hydrogen peroxide and 8-hydroxy-2-deoxyguanosine (8-OhdG) in phorbol ester (TPA) stimulated HL-60 cells. It was found that the flavone genistein was the most potent inhibitor of hydrogen peroxide production, followed by apigenin. All flavones used in the study were able to inhibit 8-OhdG formation in HL-60 cells.

The presence of these two flavones in dandelion leaf are likely to give this herb antioxidant properties. Hu and Kitts (2003) investigated the antioxidant activities of solvent-fractionated dandelion flower extracts *in vitro*, using a DPPH assay, by measuring peroxyl radical and Cu^{2+} induced peroxidation of liposome, and assessing cupric-ion induced human LDL oxidation, among others. They found that dandelion flower extract exhibited significant free radical scavenging capacities for the DPPH radical, that peroxidation of liposome was suppressed by the addition of the dandelion flower extract, similar to Trolox and luetolin-7-glycoside at concentrations of 1μg/ml.

For the cupric-ion induced hLDL oxidation, only high concentrations of dandelion flower extract prevented the formation of conjugated dienes, consistent with decreased oxidation.

The authors concluded that dandelion flower fractions possess antioxidant properties and attributed this, in part to luteolin and luteolin 7-glycoside.

Hagymasi *et al* (2000) examined the effects of natural extracts of dandelion and found reduced lipid peroxidation in microsomal fractions of rat liver. Cho *et al* (2002) investigated the antioxidant effect of dandelion in diabetic rats. Hepatic

TBARS concentration and SOD activities were significantly lowered by dandelion water extract.

Analysis of milk thistle found apigenin, luteolin and silymarin in the dried plant. Levels of luteolin were lower than that of dandelion, but levels of apigenin were higher. Silymarin was identified but at low levels (0.9 mg/g) compared to luteolin and apigenin, however, the greatest concentration of silymarin is found in the seeds. Kvasnicka et al (2003) used HPLC to analyse milk thistle seeds and found a mean of 38 mg/g silymarin in the milk thistle fruits. The milk thistle used in this study was leaf and stem hence the relative low level of silymarin quantified in the herb .
Oil obtained from milk thistle seeds and silybinin was shown to reduce lipid peroxidation, through reduced ethane exhalation, and increase catalase activity in hepatic tissue of rats intoxicated with CCl_4 (Batakov, 2001). Ryzhikova *et al* (1999) found that decoctions and infusions of milk thistle suppressed MDA levels in the liver of rats.
Kvasnicka *et al* (2003) assessed the antioxidant activities of components of silymarin using the total antioxidant status (TAS) test. They found that the antioxidant power of silybin, isosilybin and silydianin were similar, but silychristin had an antioxidant power 20% higher than the others. A mixture of all components showed 50% higher antioxidant power than that of individual standards. Interestingly, they found that the higher the purity of milk thistle extracts the lower the antioxidant power. This may suggest that the extract may contain other compounds, which in synergy with silymarin, contribute to the overall antioxidant capacity.

2.4.4 Response of urinary TBARS to antioxidant supplementation

Following supplementation with both herbs urinary TBARS significantly decreased. However, although there was a significant difference between basal levels and additional dandelion and milk thistle treatments, there was no significant difference between herb treatment and no herb treatment.

A study on cows (Vojtisek 1991) investigating the effect of milk thistle on milk production and metabolism found that differences were observed in the cows two weeks after the milk thistle was removed from the diet. The effects of dandelion are said to last for 3-4 weeks (Hilton Herbs, personal communication 2002).

In both groups of horses, urinary TBARS continue to decrease in horses 1, 2 and 3 in the 'no additional herb' treatment. Horses 1, 2 and 3 in both groups received the herb first for 2 weeks. It was withdrawn from the diet and given to horses 4, 5 and 6 for another 2 weeks. During this second 2 week period horses 1,2 and 3 are assumed to be on a 'no additional herb' treatment but if, as is suggested, there is a residual effect then this could account for the fact that once the supplement had been removed from the diet the TBARS levels decreased further. The fact that this occurred consistently in horses 1, 2 and 3 in both groups gives credence to this theory and could lead to the suggestion that in horses both dandelion and milk thistle continue to have an effect two weeks after the herbs are withdrawn from the diet.

In horses 4, 5 and 6 who received the herb in the latter part of the cross over trial, the 'no herb' treatment is unaffected by residual effects. In the dandelion group the 'no dandelion' treatment for these 3 horses gives a mean urinary TBARS value

similar to basal values. Treatment with dandelion in these 3 horses reduces urinary TBARS in all cases.

Horse	Basal pmol MDA/mmol creatinine	No dandelion pmol MDA/mmol creatinine	Dandelion pmol MDA/mmol creatinine
4	184.8	343.6	/
5	490.1	83.9	57.0
6	326.2	316.3	22.6
Mean	**333.7**	**247.9**	**26.5**

In horses 4, 5 and 6 treated with milk thistle, in all cases the 'no milk thistle' treatment gives urinary TBARS levels lower than the basal levels, but again treatment with milk thistle reduces urinary TBARS further.

Horse	Basal pmol MDA/mmol creatinine	No milk thistle pmol MDA/mmol creatinine	Milk thistle pmol MDA/mmol creatinine
4	279.5	66.1	18.9
5	338.9	82.7	43.6
6	300.5	117.9	36.8
Mean	**306.3**	**88.9**	**33.1**

It is unclear why urinary TBARS decreased to such an extent in horses 4, 5 and 6 of the milk thistle group with effectively no treatment at all. Diet, exercise and management routine was consistent throughout the trial, environmental temperature was consistent and no changes in the health status of the horses were observed. It was noted that in the first week of the trial (basal levels) these horses were very reluctant to urinate into the harnesses and initially it took them a long time to urinate. In the second week of collection the horses were more accustomed to the harnesses and urinated in a shorter period of time. This will affect the concentration

of the urine samples with more concentrated urine being produced in the first week, although expressing the results as a ratio of pmol MDA/mmol creatinine should balance this out.

Treatment with dandelion and milk thistle appeared to reduce the level of urinary TBARS in horses and therefore it could be assumed that lipid peroxidation is reduced. This effect is likely to be due to the antioxidants present in these herbs. As seen in table 2.6 the horses were ingesting significant quantities of antioxidant flavonoids from the daily supplement. Very few studies have documented *in vivo* effects of supplementation and further studies are required, although the results from the current study are encouraging.

In terms of assessing the usefulness of urinary TBARS as a marker of oxidative stress, urinary TBARS in this experiment have responded to dietary intervention with antioxidants, suggesting that they are a useful measure for use in further studies.

2.4.5 Free radical scavenging activity of saliva

Saliva was successfully collected using the saliva - collecting bit. The samples were quite heavily contaminated with hay particles, which were removed effectively by centrifugation. However, hay should be removed from the horse's stable prior to further saliva collection.

The free radical scavenging activity of saliva is due to enzymes, e.g. salivary peroxidase and vitamins and minerals. Atsumi *et al* 1999 found that heating or freeze-thawing did not influence the DPPH scavenging activity of whole saliva,

indicating that the main source for scavenging the DPPH radicals is the non-enzymic fraction.

The principle antioxidant in human saliva is uric acid, which is present at concentrations similar to those in serum. Ascorbic acid, glutathione and albumin are also present in saliva but at concentrations far below those found in the circulation. Meucci *et al* (1998) found that total protein concentration and uric acid level in saliva of humans showed good correlation with saliva total antioxidant capacity, suggesting that these are major antioxidants in this fluid.

This assay was simple and straightforward and could easily be applied to equine saliva. Therefore, the DPPH assay can be used in conjunction with the measurement of urinary TBARS as a non-invasive measurement of oxidative stress.

2.4.5 Urinary NAG activity

Surprisingly, no NAG activity was detected in the urine samples. It is possible that the level of activity in the horse urine is low, or that there are low molecular weight inhibitors in the urine samples. Low molecular weight inhibitors need to be removed when measuring activity of other urinary enzymes such as Gamma-glutamyl-transpeptidase (GGT) and alanine amino peptidase (AAP) (Rothnie 1990). Although this has not been reported for NAG it may be the case in the horse. However, if inhibitors were present, the procedures to eliminate low molecular weight inhibitors by filtration through sephadex and filtration through the Microcon units seemed to be ineffective. NAG activity was therefore measured in renal tissue to determine whether or not NAG was present in the equine kidney. NAG activity

was demonstrated in the equine kidney and the optimum incubation time appeared to be 15- 20 minutes. Km values, however, were not determined.

The enzyme activity in the kidney appeared to be high, for example, an enzyme activity (U/g wet tissue) of approximately 37 has been demonstrated in the cortex of pig kidney (P. Whiting, personal communication 1999). A NAG activity of 5.6/g wet tissue has been reported in the cortex of mouse kidney (Funakawa *et al* 1984). It is therefore clear that horse kidney demonstrates appreciable NAG activity. The problem in detecting NAG in equine urine may be due to the fact that horses produce large quantities of urine in one day. Within a 24 hour period a 500 kg horse will produce between 2.85 and 11.4 litres of urine, with an average of 5.13 litres (West 1988). It may be that NAG is simply not detectable in this volume of urine. However, NAG has been detected in cattle, which also produce large quantities of urine. However, cattle lose a lot of water in faeces (75-85%) so it is likely that non – exercising horses lose more water through the kidneys than cattle. The Microcon filters were used to concentrate the urine (approximately 5 fold) to see if this would aid in the detection of NAG activity in urine.

From the 20 horses tested only two showed NAG activity in the urine. The level of NAG activity measured was low compared to other species. U/g creatinine of 146.2 has been demonstrated in male mice, although the value for female mice is around 18 U/g creatinine (Funakawa *et al* 1984). The NAG activity in healthy cattle has been reported as 0.7 – 0.9 U/g (Sato *et al* 1997) and urinary NAG activity in cows with renal disease ranged from 1.4 – 23.0 U/g (Sato *et al* 1999). Uechi *et al* (1994) reported urine NAG activity of 5.7 $\pm$ 3.4 U/g of creatinine in healthy dogs.

Horse number 20 was 26 years old, with no health problems. However, several years ago she damaged her digital tendon on her front leg. She received surgery and recovered, but has been receiving phenylbutazone ever since. The level of medication at the time of sampling was low, but she would have received high levels during her injury and subsequent surgery. Phenylbutazone is a non-steroidal anti inflammatory drug (NSAID). It is administered to reduce inflammation which it does by enhancing break down of prostaglandins and blocking their synthesis.

The recommended dose for equids is 2-4 grams per 1000lb horse for the initial 3-4 days of treatment, then 1-2 grams per day for the rest of the course. The advised daily dose for intravenous administration is 1-2 grams per day for a 1000lb horse (Campbell 1996). It has been found however that administration of only 2 grams a day for 32 days initiated necrotizing phlebitis of the portal vein (Gabriel *et al* 1962). When phenylbutazone was administered at levels up to 30mg/Kg BW per day, post mortem examination revealed mouth and tongue sores, gastrointestinal ulcerations and renal papillary necrosis (MacKay *et al* 1983). Drugs such as phenylbutazone are metabolised extensively in the liver. Liver damage may be induced by high doses of the drug, which can potentially lead to tissue accumulation and further toxicity.

The fact that this horse was treated with this drug may explain why she showed increased NAG activity in the urine.

Horse number 6 was 20 years old and was apparently being investigated for selenium deficiency. Selenium is part of the enzyme glutathione peroxidase, which protects cell membranes from free radical damage. As a correlation has been seen

between oxidative stress and NAG activity (Skrha and Hilgertova 1999) this may explain why NAG was detectable in this horse's urine sample.

All other horses tested had no medical problems and were not receiving medication. It is interesting that out of all the horses tested these were the only two that proved to have a positive result (the medical history of these horses was obtained after the NAG analysis had been carried out). This suggests that NAG cannot normally be detected in the urine of healthy horses, and was, therefore, not used further in these studies.

CHAPTER 3

AGEING AND FREE RADICALS.

3.1 The senior horse

It has been reported that the average horse lives about 25 years (Ensminger 1989). Willoughby (1975) suggested that the horse reaches puberty at about 1.5 years, maturity at 5 years and extreme old age at 53 or 54 years, which would correspond to 14, 21 and 117-119 years for humans. On this scale, the average death of horses would be 32 to correspond to a comparative age of 75 years in humans. However, because the life expectancy is usually considered to be 25, Willoughby (1975) suggested that horses die prematurely rather than from old age.

The percentage of geriatric horses in the population is increasing, it is estimated that approximately 20% of the horse population in the United States are horses over 15 years old (http;//members.tripod.com). Recent research at Glasgow veterinary school indicated that 25% of horses in Scotland and Northern England were 16 years or older. This reflects a similar result from a survey carried out at Writtle College which highlighted that nearly 30% of horses in Essex were over 16 years (T. Hollands, personal communication, 2001). Horses over 20 years old are frequently capable of athletic performance and valued as riding stock. There is very little information

available regarding the nutritional requirements of senior horses, and in many circumstances older horses do not need "senior" rations (Ralston 2002). However, exercise has been shown to increase free radical production and if older horses are to perform athletically then it is important to establish whether age related, free radical induced changes are occurring consecutively.

The purpose of this study was to investigate whether the level of lipid peroxidation, as measured by urinary TBARS, increased during ageing in horses. Other changes in urinary composition as a result of ageing were also investigated using proton NMR. Chronic kidney or liver failure is not as common in aged horses as in cats and dogs, but still can occur (Ralston 2002), so total protein in urine was also measured

3.2 MATERIALS AND METHODS

3.2.1 Animals

Free flow urine samples were collected from 77 healthy horses (26 mares, 50 geldings, 1 stallion), age range 5-38 years. The majority of the horses were performing light work and were maintained on similar diets of forage and concentrates. The samples were obtained from horses stabled throughout the UK and were collected by their owners directly into a clean plastic bucket. The samples were stored in plastic bottles (Scientific Laboratory Supplies, Nottingham) and sent by return of post, along with a completed questionnaire detailing dietary intake, exercise routine, medical details and a brief history (see appendix 3) The samples were then frozen at -18^{0}C prior to analysis.

3.2.2 TBARS

The samples were centrifuged at 3000rpm for 10 minutes to remove any sediment. A method adapted from Yagi (1976) was used to determine TBARS, using a Perkin Elmer LS 30 luminescence spectrometer at emission and excitation wavelength of 553nm and 515 nm respectively (refer to Section 2.2.2). Urinary creatinine was determined by the Jaffe reaction (Bonsnes and Taussky 1945) using a Jenway 6105 u.v./vis spectrophotometer at wavelength 500nm (refer to Section 2.2.4).

3.2.3 Total Protein

Total protein was measured in 39 samples using the Biuret method (Goa 1953), where a coloured compound is produced due to the formation of a tetracoordinated cupric ion complex with adjacent peptide groups. Briefly, 4.0 ml trichloracetic acid (110g/l) were added to 1ml urine, which was allowed to stand for 10 minutes followed by centrifugation at 3000rpm for 10 minutes. The supernatant was removed and the sediment was dissolved in 4.0 ml sodium hydroxide solution (30g/l). Benedicts qualitative reagent (0.2ml) (Sigma Aldrich, Poole, Dorset) was added and the optical density of the resulting solution was read in a uv/vis spectrophotometer (Jenway 6105) at 330nm. A range of standards (0.5-2g/l) were prepared using standard bovine albumin solution, 2g/l (Sigma Aldrich, Poole, Dorset).

3.2.4 NMR

NMR traces were obtained from the urine of 12 horses (refer to 2.2.1). Group 1 (n = 6) had a mean age of 27.7 years, while group 2 (n = 6) had a mean age of 15.2 years. In addition, samples from 25 horses of a range of ages were subsequently analysed using proton NMR technology.

3.2.5 Statistical treatment of results

Means, standard deviation and standard error were calculated. The Mann –Whitney test for large samples and the Kruskal Wallis test were used to detect differences between groups. P values of <0.05 were considered significant.

3.3 RESULTS

3.3.1 TBARS

Table 3.1: mean pmol MDA/mmol creatinine values for young and old horses

Age group	Mean value pmol MDA/mmol creatinine	Standard deviation
1 (5-15 years) n = 10 mean age 9.7 years	121.2	35.4
2 (16-38 years) n = 67 mean age 24.1 years	282.6	185.1

Table 3.2: mean pmol MDA/mmol creatinine values for 3 different age groups

Age group	Mean value pmol MDA/mmol creatinine	Standard deviation
1 (5-15 years) n = 10 mean age 9.7 years	121.2	35.4
2 (16-24 years) n = 38 mean age 20.7 years	276.7	179.0
3 (25-38 years) n = 29 mean age 28.6 years	290.4	192.4

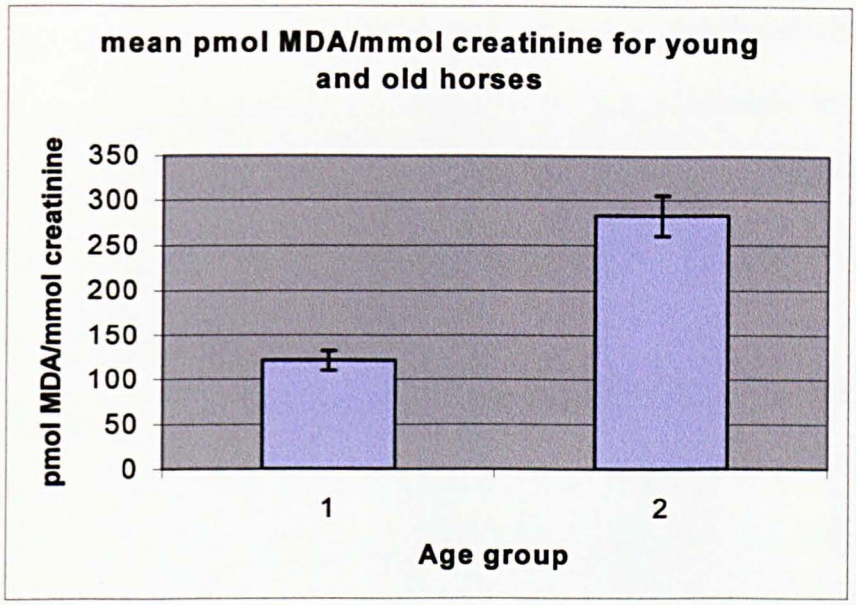


Figure 3.1 mean pmol MDA/mmol creatinine for young and old horses, displaying standard errors of 11.1 (group 1) and 22.6 (group 2)

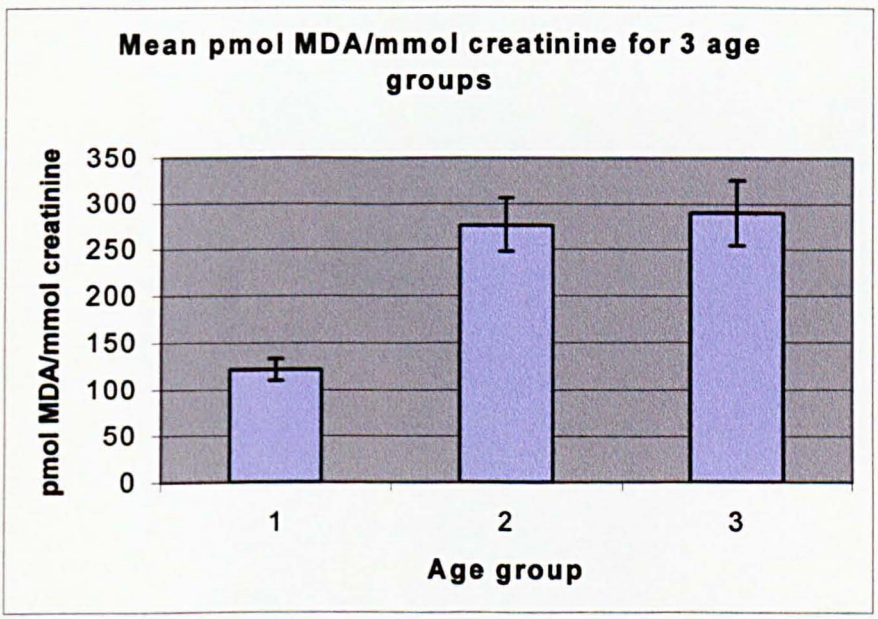


Figure 3.2 mean pmol MDA/mmol creatinine for 3 age groups displaying standard errors of 11.1 (group 1), 29.0 (group 2), 35.7 (group 3).

The horses were split into 2 age groups. Group 1, 5 – 15 years (mean age 9.7 years) and group 2, 16-38 years (mean age 24.1 years). There was a significant increase in urinary TBARs (expressed as pmol MDA/mmol creatinine) in the older group of horses when analysed using the Mann-Whitney test for large samples, with medians of 113.37 and 220.59 respectively ($p < 0.05$) (table 3.1).

The same horses were further split into 3 age groups. Group 1, 5-15 years (mean age 9.7 years), group 2, 16-24 years (mean age 20.7 years) and group 3, 24 – 28 years (mean age 28.6 years). Statistical analysis using a Kruskal Wallis test (with medians of 113.37, 218.32 and 220.59 for groups 1,2 and 3) revealed a significant difference between groups 1 and 2 and between groups 1 and 3 ($p < 0.05$) (table 3.2).

This demonstrates that there was an age-related increase in urinary TBARS in this population of horses.

3.3.2 Total Protein

Table 3.3 mean g protein/mmol creatinine for young and old horses

Age group	Mean g protein/mmol creatinine	Standard deviation
1 (7-11 years) n = 7 mean age 10 years	**0.055**	**0.029**
2 (16-32 years) n = 32 mean age 24.4 years	**0.150**	**0.33**

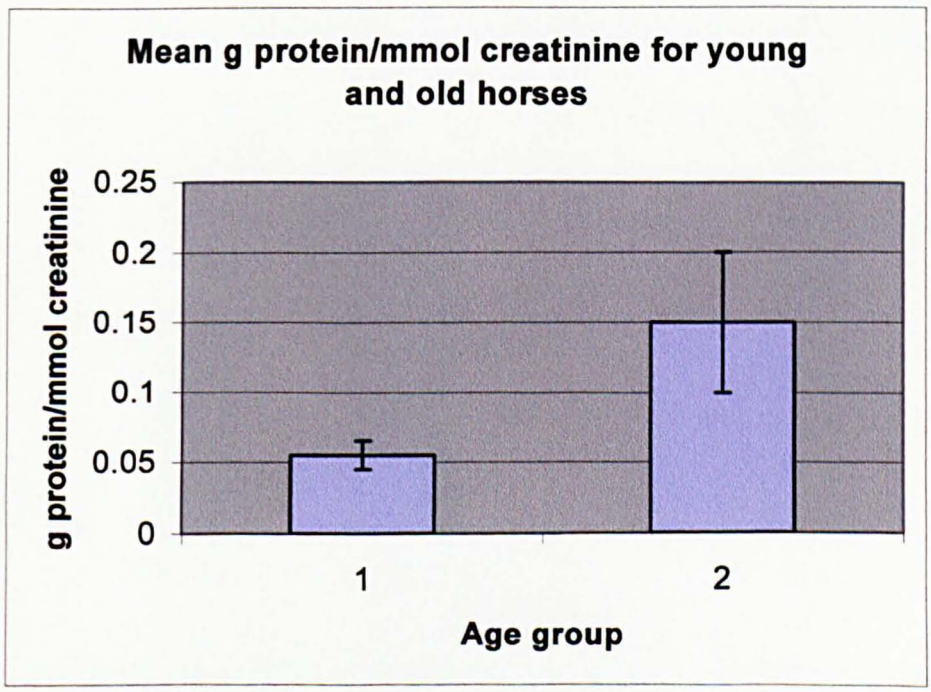


Figure 3.3 Mean g protein/mmol creatinine for young and old horses displaying standard error of 0.01 (group 1) and 0.05 (group 2)

The horses were split into 2 age groups. Group 1, 7–11 years (mean age 10 years) and group 2, 16-32 years (mean age 24.4 years), (table 3.3). There was a significant increase in the amount of protein in the urine of the older group (expressed as g protein/mmol creatinine), when analysed statistically using a Mann-Whitney test with medians of 0.048 and 0.079 respectively ($p < 0.05$).

3.3.3 NMR

Table 3.4 Group 1 and group 2 aromatic amino acid, lactate, glycine and TMAO values from NMR spectra for young and old horses

Group	AA1/Cr	AA2/Cr	Lactate/Cr	Glycine/Cr	TMAO/Cr
1 mean age 27.7 years	**2.21** s.d. 0.43	**7.34** s.d. 2.67	0.41 s.d. 0.33	0.85 s.d. 0.15	0.49 s.d. 0.29
2 mean age 15.2 years	**1.26** s.d. 0.52	**4.93** s.d. 1.29	0.66 s.d. 0.34	0.89 s.d. 0.14	0.44 s.d. 0.16

Statistical analysis using a 2 tailed student t test showed a significant difference ($p = 0.0066$) between group 1 aromatic amino acids (AA1/cr). There was almost a significant difference between group 2 aromatic amino acids (AA2/cr) ($p = 0.075$). There was no significant difference between any of the other metabolites.

Table 3.5 Group 1 and group 2 aromatic amino acid values for young and old horses from NMR spectra

Age group	AA1/Cr	AA2/Cr
1 (5–11 years) n = 5, mean age 8.4 years	1.51 s.d. 0.56	3.39 s.d. 0.67
2 (16-31 years) n = 20, mean age 23.5 years	2.09 s.d. 1.35	4.86 s.d. 2.66

Statistical analysis (Mann – Whitney U test) revealed a significant difference between the young and old groups for group 2 aromatic amino acids ($p<0.05$).

There were no other significant differences between the young and old group for any other metabolites. These results indicate that older horses appear to have higher levels of aromatic amino acids in their urine than young horses.

3.4 DISCUSSION

3.4.1 TBARS

The results of the studies presented in this chapter show that in this group of horses there appears to be an age-related change in the urinary excretion of TBARS.

Initially the horses were divided into two groups, group 1 ranging from 5 – 15 years, with a mean age of 9.7 years and group 2, ranging from 16-38 years, with a mean age of 24.1 years. The older horses had significantly higher levels of TBARS in their urine compared to the younger horses. The horses were then further sub-divided into three groups, group 1 the same as before, group 2, ranging from 16-24 years with a mean age of 20.7 years and group 3 ranging from 25-38 years with a mean age of 28.6 years. Statistical analysis using the Kruskal Wallis test showed that group 1 had significantly lower levels of urinary TBARS than groups 2 and 3 and that there was no significant difference between groups 2 and 3.

The reason for further sub-dividing the group was to try and establish more clearly at what age the increase in urinary TBARS occurred, whether it was a consequence of extreme old age or if it occurred in 'middle age'.

The results suggest that between the ages of 10 and 20 years an increase in urinary TBARS was demonstrated. As the ages in the younger group ranged from 5 – 15 years,

it may be sensible to assume that horses above the age of 15 years have higher levels of urinary TBARS.

One of the problems with carrying out a study of this nature lies in the fact that it is difficult to obtain a sufficient number of samples reflecting a range of ages. For this reason the general public were involved in this trial and were invited to respond to adverts placed in popular equestrian magazines. Owners who expressed an interest were sent a sterile plastic bottle and a questionnaire to complete (appendix 1) and asked to send any collected urine by return of post, which was then frozen, prior to analysis. The owners were asked to state the date and time of collection so any samples arriving that were more than 48 hours old were discarded.

MDA measurements done at different times for the same human urine sample frozen at -40 °C indicated no significant difference in the MDA concentration for at least 3 months storage (Guichardant *et al* 1994). A similar test was performed on a sample of horse urine frozen at – 18 °C and this indicated the same.

Guichardant *et al* 1994 also noted that the MDA complexes in urine were stable for at least 48 hours.

Any samples from horses that were receiving veterinary treatment or who were reported to suffer from chronic illnesses were also discarded.

Overall 88 samples were received. 3 samples were discarded from horses that were suffering from laminitis, 2 were discarded from horses with Cushings syndrome and 6 samples were discarded, as they were more than 48 hours old.

An obvious disadvantage is the fact that there was no control over the population of horses and the horses used in this study were from a wide range of backgrounds. Also, it is a fairly crude method of collection, with deterioration of the sample being inevitable. However, most samples arrived within 24 hours of collection and the majority of the samples were collected in this way, so at least a comparison could be made. Every effort was made, using the information provided by the owner in the questionnaire, to control the samples used as far as possible.

Most of the horses in the study were performing light work (65 out of 77). Although exercise does increase lipid peroxidation (Kosugi *et al* 1994) it is unlikely that the light work loads of these horses would significantly increase lipid peroxidation. This was demonstrated in Section 2.2.2 where no significant difference was noted between horses in light work and non-working horses.

In addition, the dietary intakes of the horses were not controlled. However, all horses were receiving a balanced diet and no horses were receiving antioxidant supplements.

Bearing the limitations of this study in mind the results are similar to those found by other workers, in other species. A study by Tokunaga *et al* (1998) measured serum concentrations of lipid peroxide and antioxidants in elderly, middle aged and young people and they found that lipid peroxides in the elderly group were significantly higher than those in the young group. They also noted that levels of superoxide dismutase, glutathione peroxidase and albumin were lower than those in the young group. Bhagwat (1997) measured serum lipid peroxides and erythrocyte superoxide dismutase activity, also in humans. Serum lipid peroxide levels were found to be increased

progressively with age, while erythrocyte SOD was found to be decreased with age. In this study a strong positive correlation was found between serum lipid peroxide and age ($r = 0.959$) and erythrocyte SOD was inversely correlated with age ($r = - 0.84$). Leeuwenburgh *et al* (1994) measured the glutathione content and antioxidant enzyme activities in skeletal muscle of young, adult and male rats. They found that in the soleus muscle, glutathione content increased markedly with age, but did not alter in the deep portion of the vastus lateralis muscle. Activities of glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase and glutathione sulphur transferase were increased significantly with ageing in both soleus and vastus lateralis muscle. In addition, muscle lipid peroxidation was significantly increased with ageing in both types of muscle. It was suggested that these changes most likely reflected an enhanced oxidative stress in senescent muscle. Leeuwenburgh *et al* (1994) showed that there was a significant increase in lipid peroxidation in ageing muscle, despite elevated levels of antioxidant enzymes. This suggests that a natural adaptation of antioxidant systems is not sufficient to prevent enhanced lipid peroxidation during ageing. Consequently, if this is the case, antioxidant supplementation may be beneficial (Leeuwenburgh *et al* 1994).

Free radicals are produced by enzymes as part of the recognition process of foreign material, by activated phagocytotic neutrophils. Binding of foreign material activates a neutrophil membrane-bound enzyme that initiates the oxidation of NADPH to NADP+ within the cell, and the transfer of those electrons to oxygen, reducing it to $O_2^{\bullet -}$ on the

external surface (Gutteridge and Halliwell 1994, Halliwell and Gutteridge 1990). Phagocytes are activated by degenerative conditions such as inflammation of joints and other arthritic changes, which may be present in the older horse.

Another factor that can influence oxidative stress is body condition. During the ageing process the proportion of fat to lean tissue increases. If free radical production is increased in the senior horse then unsaturated fats, prone to attack by free radicals, may become oxidised leading to increased excretion of markers of lipid peroxidation, such as TBARS.

It could be argued that an increase in urinary TBARS observed in these horses is a consequence of body condition rather than age, as obesity has been linked to increased oxidative stress. However, although the ratio of lean tissue:fat is altered in the senior animal, many of the owners of the horses used in this study commented in the questionnaires that keeping condition **on** their senior horse was a problem. Weight loss is a common problem in senior horses, although it is often related to poor dentition (Ralston, 2002). Nevertheless, it is likely that most of the senior horses in this study were not in the obese category.

In addition, the horses in the present study aged greater than 30 years, with the exception of only two horses, had lower levels of TBARS than the mean. [a]Pampiona *et al* (2000) found a significant negative correlation between the degree of fatty acid unsaturation in heart tissue and maximum life span potential (MLSP). Unsaturated fatty acids are the tissue macromolecules most sensitive to oxidative damage and the study

also showed that the sensitivity to lipid peroxidation and the *in vivo* lipid peroxidation in the heart were also negatively correlated with MLSP across species.

Another study ([b]Pamplona *et al* 2000) investigated the relationship between fatty acid composition, protein lipoxidation and MLSP by analysing liver fatty acids and proteins from animals with a range of MLSP. The results showed that the peroxidisability index of fatty acids and the sensitivity to *in vitro* lipid peroxidation were negatively correlated with MLSP. The authors proposed that a low degree of fatty acid unsaturation may have been selected in long-lived mammals to protect their tissue lipids and proteins against oxidative damage. This may occur within species and it could be the case that these very old horses have a low degree of tissue fatty acid unsaturation compared to the other horses studied.

Among other changes associated with ageing is a decline in glucose tolerance (Preuss, 1997). Changes in glucose/insulin metabolism are associated with increased free radical formation. Facchini *et al* (2000) suggest that hyperinsulinaemia could affect antioxidant enzymes and free radical generation and therefore increase oxidative stress. In adipocytes cultured *in vitro*, insulin has been shown to increase the production of hydrogen peroxide (Ceriello 2000). Ceriello (2000) suggested that insulin resistance may cause elevated plasma free radical concentrations, which in turn might be responsible for a deterioration in insulin action.

Hyperinsulinaemia is a commonly observed characteristic of insulin resistance and it may be that there is an age-related change in glucose tolerance in this population of

senior horses, which is not related to body condition. In an early study of geriatric horses (from Ralston 2002) over 70% of the horses over the age of 20 years had at least sub-clinical signs of pituitary/thyroid dysfunction. One of the characteristic signs of Cushings syndrome is glucose intolerance. Garcia and Beech (1986) found resting hyperglycaemia in horses suffering from pituitary adenoma and a delayed return of glucose values to baseline following intravenous glucose injection. These horses also showed resting hyperinsulinaemia with a poor response to the glycaemic stimulus.

The results from the present study seem to be consistent with the proposal that the level of oxidative stress to various tissues increases during the ageing process. This could be due either as a result of increased free radical production or decreased efficiency of the protective antioxidant systems. In general, the results are consistent with increased lipid peroxidation in horses above the age of 15 years and therefore supplementation of antioxidants may be of benefit.

3.4.2 Total Protein

The results demonstrate a significantly higher level of total protein in the urine of older horses, compared to younger horses.

The proteins present in normal urine are derived from the plasma proteins and also from the urinary tract (Rothnie 1990). Increased excretion of proteins in urine may occur as a result of renal disease or in other diseases affecting renal function. This is due to changes in the basement membrane structure of glomeruli allowing increased passage

of proteins. Increased glomerular permeability with the presence of proteins of MW 60,000 to 500, 000 in the glomerular filtrate is associated with nephritic syndrome (Rothnie 1990). In general, the more severe the changes, the larger the size of proteins appearing in the filtrate. Destruction of nephrons is a feature of many types of kidney disease and results in a reduction in glomerular filtration rate (Walker 1990). This results in an increased urinary excretion of proteins such as albumin and transferrin. These proteins are present in plasma and would normally be substantially retained by the glomerulus.

Increased proteinuria may also be due to impaired reabsorption of protein in the tubules. (Varley *et al* 1980). Tubular proteinuria is the failure of the tubules to reabsorb proteins present in the plasma at physiological concentrations, which have been filtered through an intact glomerulus (Rothnie 1990). An increase in the excretion of low molecular weight proteins is a sensitive indicator of renal tubular damage because in health reabsorption is almost complete.

In addition, an increase in the activity of urinary enzymes can indicate insult to the kidney. For example, elevated N-acetyl-β-D-glucosaminidase (NAG) enymuria is associated with conditions such as glomerulonephritis, nephrotic syndrome, rheumatoid arthritis and infection (Rothnie 1990). NAG activity has also been seen to increase with age (Oba *et al* 1999). In addition, increased urine activities of the brush border enzyme Alanine aminopeptidase (AAP) is associated with acute renal failure and hyperthyroidism (Rothnie 1990).

Increased urinary concentration of a protein normally present in the plasma can result even when the kidneys are normal. The α_1-globulins, α_1-antitrypsin and α_1-acid glycoprotein are present in inflammatory states and in cancer and are known as acute phase reactive proteins. They have a relatively low molecular weight and are excreted into the urine especially when there is increased concentration in the plasma (Varley *et al* 1980).

Many of the older horses probably suffered from some degree of degenerative joint disease, which may cause an increase in plasma proteins such as the α_1-globulins which are increased during inflammatory conditions.

Ralston (2002) found that old mares with pituitary dysfunction had lower blood vitamin C than did unaffected or younger mares, which could explain, in part, the increased susceptibility to infections observed in older horses (Ralston 2002). This increased susceptibility to infection could explain an increase in urinary protein excretion if there is an increase in plasma α_1-globulins. In addition pituitary dysfunction can cause hypertension which can cause damage to the glomerulus, leading to increased protein excretion. However, in the current study none of the horses were exhibiting overt clinical signs such as hypertension.

It is the case that urinary nitrogen excretion increases with an increase in dietary protein (Frape 1998). Although dietary intake could not be controlled in this study, all horses were receiving similar diets of forage and commercial concentrates. Crude protein levels in forage vary according to factors such as variety of grass and stage of growth

but most grass, grass hays or haylage will provide approximately 10 – 5 % protein (DM). Legume forages such as alfalfa have a higher protein content of approximately 16-17% (DM). Alfalfa was included in the diet of some of the older horses, but only as part of the ration and not as the main forage and often as a replacement for the concentrate part of the ration. The protein content of concentrates is less variable, with a protein content of approximately 10 % protein (DM) in the concentrates that were fed to most of the horses in this study.

The level of proteinuria has been found to increase with exercise intensity (Schott *et al* 1995). The majority of horses in this study were performing a similar level of light work and in fact it was the younger horses that were most likely to be working harder. It is therefore unlikely that exercise has caused increased proteinuria in the senior horses.

In the current study emphasis was placed on collecting samples from healthy horses only. However, although renal disease may not be overt, some of the older horses may have been suffering from some degree of renal insult and dysfunction, therefore resulting in increased protein excretion.

A single dose of puromycin aminonucleoside (PAN) given to rats induced ultrastructural glomerular changes and a nephrotic syndrome similar to that seen in humans, resulting in increased proteinuria (Cutler *et al* 1999). In addition, free radicals are thought to be mediators in the induction of glomerular injury and proteinuria. Srivastava *et al* (1995) determined the MDA content of glomeruli and its urinary

excretion in rats administered PAN. Increased proteinuria was observed in rats 4 days following PAN injection and glomerular and urinary MDA was significantly higher than control animals. Shibouta *et al* (1991) found that a single intraperitoneal injection of PAN induced proteinuria in rats and increased MDA levels in plasma, urine and renal cortex. Gwinner *et al* (1997) directly examined the generation of reactive oxygen species in glomeruli isolated from rats with PAN induced nephrosis, using chemiluminescence techniques. Levels of reactive oxygen species were increased in glomeruli following PAN injection and the hydroxyl radical and hydrogen peroxide appeared to be predominantly involved in the initial increase of reactive oxygen species. Nine days after induction of nephrosis, superoxide and hydroxyl radicals were found to contribute to increased reactive oxygen species. In addition, catalase and glutathione peroxidase activities declined following PAN injection. The authors concluded that the increase in glomerular reactive oxygen species was sufficient to induce oxidative glomerular injury and that the glomerulus may not necessarily respond to oxidative stress with the up-regulation of antioxidant enzymes.

Dileepan *et al* (1993) investigated the response of isolated glomeruli to superoxide anions generated by xanthine and xanthine oxidase or by activated macrophages. Following exposure of glomeruli to superoxide, albumin permeability was significantly increased. Further, the increase in albumin permeability was prevented by superoxide dismutase.

Ricardo *et al* (1994) administered antioxidants to PAN treated rats. Superoxide dismutase, dimethyl thiourea and a combination of α-tocopherol and ascorbic acid all

reduced proteinuria, with the greatest effect demonstrated by α-tocopherol and ascorbic acid (90 % reduction). Analysis of kidneys using transmission electron microscopy found that the rats receiving antioxidants contained more glomerular epithelial cell filtration slits per unit length of glomerular basement membrane than rats treated with PAN alone.

It has also been found that there are considerable differences in the antioxidant-oxidant balance between the glomerulus and the proximal tubule, with greater activity of antioxidant enzymes being evident in the proximal tubules. This suggests that the glomerulus may be more susceptible to oxidative stress (Gwinner *et al* 1998).

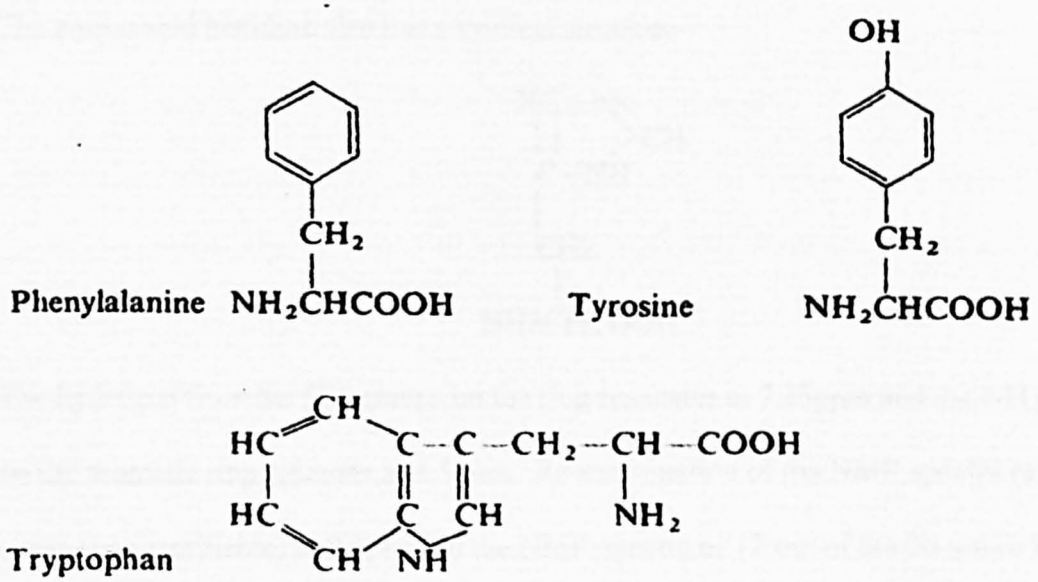
The results from the current study indicate increased TBARS excretion in urine and increased proteinuria in senior horses and it is possible that these two events are linked, considering the observations discussed above. Increased oxidative stress to ageing tissues may cause glomerular injury, thus leading to increased protein excretion.

3.4.3 NMR

Urine is a complicated mixture of compounds and many metabolites can be identified and quantified using ^{1}H NMR spectroscopy without separation or derivatisation (Bales *et al* 1984). This proved to be a clean and simple technique particularly useful in this instance, where any differences in urine composition of senior horses compared to younger horses were unknown. This 'snapshot' information is extremely useful.

The only difference detected in the urine of older horses using ^{1}H NMR was that older horses appeared to have higher levels of aromatic amino acids in their urine. The first

group of senior horses tested had higher levels of group 1 aromatic amino acids in their urine, while the second group showed higher levels of group 2 aromatic amino acids. Aromatic amino acids are phenylalanine, tyrosine and tryptophan.



McDonald *et al* (1995)

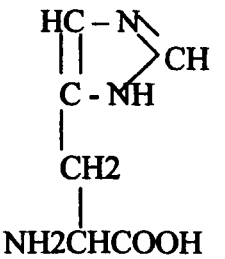
The aromatic hydrogen atoms on phenylalanine resonate in the range 7.3-7.4 ppm and there are different peaks for the ortho, meta and para hydrogen atoms. There are 2 unique proton chemical shifts on the tyrosine ring near 7 ppm and tryptophan resonates at 7.1-7.2 and also at 7.6-7.8 ppm

(http://www.isat.jmu.edu/users/klevicca/isat454/NMRChemscape.doc).

The peak referred to as group 1 occurs around 7.8 so this relates to the aromatic amino acid tryptophan. The first group of senior horses therefore appeared to excrete higher levels of the aromatic amino acid tryptophan. The second group of senior horses showed higher levels of the substances resonating at 7 – 7.5ppm (referred to as group

2), but no difference between young and old horses for group 1 peaks. Therefore, this second group of horses appeared to be excreting higher levels of phenylalanine and tyrosine.

The amino acid histidine also has a cyclical structure



The hydrogen from the CH groups on the ring resonates at 7.25ppm and the NH groups on the aromatic ring resonate at 8.5ppm. Re-examination of the NMR spectra revealed peaks (unquantifiable) at 8.5ppm on the NMR spectra of 12 out of the 20 senior horses (age range of these 12 horses, 20-32 years). This peak was not present or tiny in comparison in all the spectra of the horses in the young group (refer to appendix 6) This suggests that the senior horses are also excreting higher levels of histidine compared to younger horses. It has been suggested that over 70% of the horses over the age of 20 years had at least sub-clinical signs of pituitary/thyroid dysfunction (Ralston 2002). Pituitary dependent Cushing's disease is caused by a functional pituitary tumour that stimulates the pituitary gland to produce excess cortisol. Excess cortisol causes increased protein catabolism. 3-methyl histidine and tyrosine are markers of protein catabolism. The increased excretion of aromatic substances resonating in the region 7 – 7.5 ppm, and increased proteinuria in these horses supports the theory that pituitary dysfunction may be evident in senior horses.

Generalised renal tubular damage may interfere with tubular reabsorption of amino acids (Varley *et al* 1980) which would also lead to increased levels in the urine. NMR urinalysis performed after renal damage in experimental animals showed that proximal tubular injury was associated with glucosuria, aminoaciduria, lactic aciduria along with reduced excretion of TCA cycle intermediates. Medullary damage led to the appearance of trimethylamine-N-oxide (TMAO) and dimethylamine, followed by increased excretion of acetate and succinate (Bairaktari *et al* 1998).

Bales *et al* 1984 used ^{1}H NMR spectroscopy to examine the urine of a man known to have kidney damage following industrial exposure to cadmium. Prolonged exposure to cadmium can result in functional and structural damage to the proximal tubules. The ^{1}H NMR spectrum of this individual showed high concentrations of amino acids, including alanine, valine, threonine, and lysine. A survey conducted in 1975 – 1976 (Penumarthy *et al* 1980) found that elevated tissue levels of cadmium were observed in horses as compared to other species. The median concentration of cadmium in the kidney was 4 times higher in the horse compared to pigs, cattle and dogs. These findings were supported by Salisbury *et al* (1991) who again found higher levels of cadmium in liver and kidney of horses, compared to other species. Baldini *et al* (2000) found that cadmium concentrations in equine muscle, liver and kidney were related to life span and increased with age. It does appear that there is specific cumulation of cadmium in equine tissue so it is possible, therefore, that the increased excretion of amino acids seen in the older horses is due to proximal tubule damage caused by cadmium accumulation.

A major advantage of using NMR spectroscopy is that it provides information about the abnormal pattern of endogenous metabolites that characterise the location of any injury in renal tubules and reveals alterations in unusual metabolites that are not commonly measured. As the only difference detected was increased aminoaciduria in the urine of senior horses it could be suggested that this is consistent with proximal tubular insult. However, other factors can effect aminoaciduria. Liver disease also results in a marked increase in plasma amino acids. This exceeds the capacity of the normal renal tubule to absorb them and so they will be excreted in the urine. (Varley *et al* 1980). Reduced liver function can be a problem in older horses and it is usually progressive. It may be as a result of repeated toxic insults from plant-derived pyrrolizidine alkaloids, from parasites or from calculi within the bile duct causing obstruction.

In the case of both protein and amino acid excretion the magnitude of the alteration observed in the older horses are not consistent with the presence of an overt clinical condition. However, the increases are significantly different to values found in the younger horses therefore suggesting that subtle changes in renal, hepatic and endocrine functions are evident in the senior horses as well as increased lipid peroxidation. Increased protein excretion and increased lipid peroxidation may well be linked and be signs of oxidative stress to tissues. The fact that administration of antioxidants to animals suffering from PAN induced nephrosis decreased proteinuria and ameliorated damage to glomerular epithelial cell foot processes (Ricardo *et al* 1994) suggests that possibly for the senior horse an increased intake of antioxidants would be beneficial.

The increased excretion of aromatic amino acids, in particular tyrosine and histidine also suggests evidence of pituitary dysfunction, and this further reinforces the value of NMR for detecting subtle metabolic changes before clinical signs become apparent.

CHAPTER 4

To determine the effect of vitamin E supplementation on lipid peroxidation in exercising horses.

4.1 VITAMIN E

Vitamin E is a fat soluble vitamin and eight naturally occurring forms are known and can be divided into two groups according to whether the side chain of the molecule is saturated or unsaturated (McDonald *et al* 1995). The most biologically active and widely distributed molecules are the saturated forms of the vitamin, α-, β-, γ- and δ-tocopherol. The β, δ and γ forms approximately 45, 0.4 and 13 per cent of the activity of the α form respectively (McDonald *et al* 1995) which is the most effective antioxidant (Kamal-Eldin *et al* 1996).

The bioavailability of vitamin E is largely dependent upon the digestion and absorption of dietary fat. Vitamin E is absorbed along with fatty acids in the small intestine and is incorporated into chylomicrons and transported throughout the body via the lymphatic system. The chylomicrons are then broken down by lipoprotein lipase and the vitamin E equilibrates with both High-Density Lipoproteins (HDL) and Low-Density Lipoproteins (LDL) (Groff *et al* 1995). From the HDL all circulating lipoproteins eventually receive vitamin E as HDL readily transfers vitamin E to the other lipoproteins at a rate equivalent to 10% of the plasma vitamin E content per hour

(Traber 1999). These lipoproteins have the ability to transfer vitamin E to the tissue as needed.

The vitamin E remaining in the chylomicron becomes a chylomicron remnant and travels back to the liver. Once in the liver the vitamin E is packaged into Very Low Density Lipoproteins (VLDL) and excreted back into the circulation. α tocopherol constitutes over 80% of the total vitamin E packaged into VLDL and secreted by the liver (Traber 1999). As the VLDL are broken down by lipoprotein lipase, Low Density Lipoproteins (LDL) are formed and from these lipoproteins vitamin E is transferred to HDL and eventually incorporated into either circulating lipoproteins or adipose tissue. 90% of total body vitamin E is found in adipose tissue (Traber 1999).

Vitamin E is excreted mainly via bile, urine, faeces and the skin. The vitamin is oxidised and forms hydroquinones, which are then conjugated with glucuronic acid to form water soluble glucuronate conjugates. Once formed the glucuronate conjugate can be excreted into bile or further degraded in the kidneys and excreted in the urine. However, under normal physiological conditions, urinary excretion seems to be quantitatively unimportant since it represents only 1% of an administered dose (Bramley *et al* 2000). A large proportion of ingested vitamin E is unabsorbed so faecal excretion is a main route for vitamin E elimination.

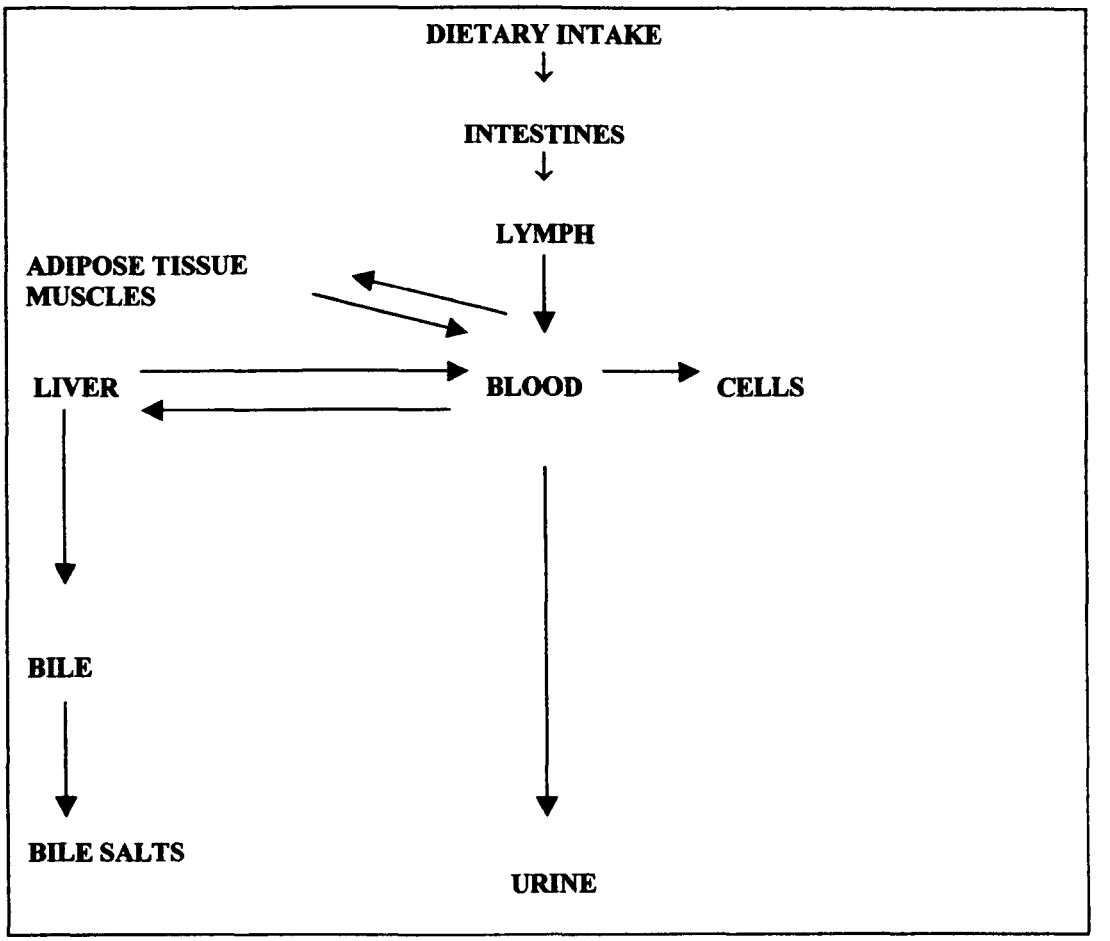


Figure 4.1 metabolism of Vitamin E

4.1.2 Function

Vitamin E is an integral part of cellular membranes whose main role is as an antioxidant. Within cells and organelles vitamin E is the first line of defence against lipid peroxidation. The major store of membrane-bound vitamin E is in the inner mitochondrial membrane, the site of the electron transport system (Evans 2000).

α tocopherol donates a phenolic hydrogen atom to peroxyl radicals to form an α - tocopheroxyl radical. This radical is fairly stable because the unpaired electron on the oxygen atom is delocalised throughout the aromatic ring (Bramley *et al* 2000). The α - tocopheroxyl radical does not react with membrane PUFA and is either reconverted to α-tocopherol or it reacts with a second radical to form a non-radical species.

Peroxyl radicals react with α tocopherol approximately 10,000 times faster than they react with PUFA (Bramley *et al* 2000), so it is more likely that an oxidative chain reaction will be quenched than propagated. Also, the hydrophobic 13-carbon side-chain of α-tocopherol seems to allow it to position itself close to those fatty acids at risk of oxidative damage, within a membrane, with the phenolic hydroxyl group being positioned at or near the surface of the membrane (Bramley *et al* 2000).

4.1.3 Vitamin E and Exercise

Hartmann *et al* (1995) demonstrated decreased MDA production in human subjects consuming 1200 mg of vitamin E for 14 days prior to a run to exhaustion. Sumida *et al* (1989) showed that vitamin E reduced pentane production and lipid peroxidation products from the mitochondria in vitamin E supplemented humans.

Reddy *et al* (1998) found that vitamin E and or selenium deficiency resulted in the generation of free radicals as measured by electron spin resonance in lung tissue of rats, which was additionally increased when the rats were subjected to a single bout of exhaustive exercise. No free radicals however were measured in the lung tissue of vitamin E and Se supplemented rats when subjected to a similar exercise programme.

Meydani *et al* (1993) found that vitamin E supplementation significantly increased plasma α tocopherol in plasma and skeletal muscle in humans. In addition, all vitamin E supplemented subjects excreted significantly less urinary TBARS after eccentric exercise at 75% maximum heart rate.

The aim of the studies conducted in this chapter was to investigate whether a vitamin E supplement given to exercising horses would decrease lipid peroxidation.

4.2 MATERIALS AND METHODS

4.2.1 Pilot Study

Three geldings and one mare were used, aged between 11 and 14 years, mean bodyweight 598 kg, range 566kg – 622kg. The horses were performing a similar level of work (3 hours per day). The exercise consisted of lessons in the school and was a mixture of flat work and jumping. The horses were receiving similar diets and none of the horses were receiving any medication or supplements. For details of horses and diets refer to appendix 4.

Urine samples were collected from each horse, post exercise, using an Equisan urine and faeces collection harness, prior to dietary supplementation.

Each horse received a supplement of α tocopherol acetate (Sigma Aldrich, Poole, Dorset) at a level of 4mg/kg bodyweight for a period of 1 week and urine samples were collected post exercise at the end of this period.

The urine samples were centrifuged at 3000rpm for 10 minutes, prior to analysis, to separate any sediment.

All urine samples were analysed for TBARs using a fluorimetric method (Perkin Elmer LS 30) adapted from Yagi 1976 (refer to Section 2.2.2).

All samples were also analysed for creatinine using the Jaffe reaction (refer to Section 2.2.4).

4.2.2 Treadmill trial

Seven thoroughbred geldings aged between 12 – 19 years of similar body condition (3 on scale 0 – emaciated, 5, obese, Carroll and Huntingdon, 1988), mean bodyweight 578 kg, range 522-626kg were used in a crossover trial over a 35 day period. They were maintained on a diet of haylage and concentrates, supplemented with salt and were kept at grass. For details of horses and diets refer to appendix 5.

In week 1 of the trial the horses were maintained on their normal rations and underwent a standard exercise test (SET) on a high speed treadmill on days 1, 3 and 5. All horses were accustomed to using the treadmill and were at a similar level of fitness.

Phase	**Treadmill angle**	**Speed (m/s)**	**Duration (min)**
Warm up	6^{o}	2	3
Warm up	6^{o}	4	3
SET	6^{o}	2	3
SET	6^{o}	4	3
SET	6^{o}	5	3
SET	6^{o}	8	3
Cool down	6^{o}	4	3
Cool down	3^{o}	2	3
Cool down	3^{o}	2	3

Figure 4.2 The high speed treadmill

A fan was placed in front of the treadmill to direct airflow onto the horses during exercise.

Heart rate measurements were taken using Polar heart rate monitor equipment, with equine electrodes. Data were recorded in two forms; one transmitter recorded RR data (every beat) throughout the test period, whilst the other transmitter recorded heart rate every 5 seconds throughout the entire exercise duration. In addition, the heart rate was taken manually during the last fifteen seconds of each step.

Horses were weighed on a weighbridge before and after each SET in order that total water loss (kg) from evaporative sources could be estimated. Rectal temperatures (°C) were recorded before and after each SET. Ambient temperatures were also recorded. During every exercise test the following were collected from all horses:

4.2.2 (i) Saliva

Saliva was collected during the test and cool down phases using a bit designed to collect saliva (refer to Section 2.2.5). The samples were stored in 1.5 ml Eppendorf tubes and frozen at –18°C prior to analysis.

4.2.2 (ii) Sweat Collection Pilot Study

For 1 week prior to the trial, while the horses were being conditioned on the treadmill, several methods were used to collect sweat, which was subsequently analysed for TBARS using the method adapted from Yagi (1976) (refer to 2.2.2).

i) Sweat was scraped directly off the horse's body, mainly from the abdomen, into a plastic universal.

ii) Small (50mm) and large (125mm) Whatman 541 hardened ashless filter papers were taped directly to the horse's neck.

iii) A plastic cover was used to hold the filter paper (large and small) to the horse's neck.

iv) Volumes of 50ml and 25ml of distilled water were used to elute the sweat from the filter paper.

4.2.2 (iii) Sweat Collection

A plastic cover held a piece of pre-weighed Whatman 541 hardened ashless filter paper (125mm)to the horse's neck.

Figure 4.3 sweat collection using Whatman 541 hardened ashless filter paper, held in place by the plastic sleeve.

Sweat was collected during the test phase only. After the test the filter paper was weighed and washed with 25 ml of distilled water. The eluent was stored in polypropylene vessels and frozen at –18°C prior to analysis.

4.2.2 (iv) Urine

Urine was collected post exercise either using Equisan urine and faeces collection harnesses or in a plastic bucket. 45ml of urine were added to 5ml glacial acetic acid, the sample was kept in the dark and analysed within 2 hours for vitamin C content. Another sample was centrifuged at 3000 rpm for 10 minutes and frozen in a polypropylene vessel.

4.2.3 Supplementation

Following the exercise test on day 5, 4 horses received a vitamin E supplement in the form of α tocopherol acetate at a level of 3 mg/kg bodyweight per day. All horses then underwent the SET on days 15, 17 and 19. Following the test on day 19 the supplement was removed from the diet of the 4 horses and the remaining 3 horses received the supplement in their diet. The horses were exercise tested again on days 29, 31 and 33.

4.2.4 Analysis

4.2.4 (i) Saliva

The free radical scavenging activity of the saliva samples was established by measuring the reduction of the free radical 1,1 – diphenyl-2-picrylhydrazyl (DPPH) when exposed to saliva, (refer to Section 2.2.5).

4.2.4 (ii) Sweat

The diluted sweat samples were analysed for TBARS using the method adapted from Yagi (1976), (refer to 2.2.2), using a Perkin Elmer Luminescence Spectrometer LS 30.

4.2.4 (iii) Urine

The acidified urine samples were analysed for vitamin C content using the method of Harris and Ray (1935).

40 mg of 2,6-dichlorophenolindophenol was dissolved in 100ml of distilled water. The volume of urine required to decolourise 0.5 ml of dye was recorded.

Urinary ascorbic acid was calculated as

$$\text{Urinary ascorbic acid (mg/l)} = \frac{111}{\text{ml urine required}}$$

The urine was also analysed for TBARS using the method adapted from Yagi (1976), (refer to section 2.2.2), using a Perkin Elmer Luminescence Spectrometer LS30.

Creatinine was also measured using the Jaffe reaction (refer to section 2.2.4).

4.3 RESULTS

4.3.1 PILOT STUDY

Table 4.1 TBARS in equine urine, pre and post vitamin E supplementation expressed as pmol MDA/mmol creatinine

Horse	**Pmol MDA/mmol creatinine Basal**	**Pmol MDA/mmol creatinine Vitamin E**
1	645	251
2	449	94
3	370	157
4	636	33
Mean	**525 s.d. 137.26**	**134 s.d. 93.13**

The data was tested using a matched pairs t test which showed that the levels of TBARS decreased significantly ($p<0.05$) when vitamin E was supplemented in the diet.

4.3.2 TREADMILL TRIAL

4.3.2 (i) Heart Rate

Table 4.2 Peak heart rates during the test phase of the SET (beats per minute)

Horse	Week 1	Week 2	Week 3
Barty	193	195	189
Buddy	223	208	211
Lloyd	212	201	211
Salesman	184	185	185
Jerome	186	199	187
Scotty	182	195	184
Stamp	181	186	182

The peak heart rates ranged from 181 – 223 beats per minute and were achieved during the 8 m/s phase of the SET.

Two horses in this study reached heart rates of over 200beats/min. The remaining 5 horses had a mean maximum heart rate of 188 beats per minute.

Table 4.3 Mean RR values (ms) for the test phase of the SET

Horse	Speed m/s	Basal (milliseconds)	Vit E treatment (milliseconds)	No Vit E treatment (milliseconds)
Barty	2	603 ± 29	614 ± 75	636 ± 33
	4	460 ± 40	440 ± 34	474 ± 31
	5	387 ± 43	385 ± 36	403 ± 14
	8	325 ± 35	328 ± 44	329 ± 22
Buddy	2	505 ± 29	596 ± 40	636 ± 26
	4	413 ± 33	466 ± 62	579 ± 46
	5	369 ± 43	405 ± 56	464 ± 35
	8	312 ± 65	307 ± 51	317 ± 20
Lloyd	2	472 ± 38	547 ± 27	486 ± 25
	4	380 ± 31	406 ± 31	371 ± 21
	5	331 ± 27	351 ± 26	317 ± 20
	8	297 ± 34	326 ± 57	291 ± 16
Salesman	2	563 ± 18	588 ± 43	587 ± 105
	4	456 ± 24	477 ± 70	476 ± 158
	5	418 ± 14	428 ± 21	408 ± 36
	8	356 ± 16	398 ± 66	226 ± 61
Jerome	2	602 ± 92	634 ± 68	587 ± 37
	4	461 ± 79	498 ± 46	465 ± 49
	5	424 ± 71	489 ± 38	409 ± 53
	8	347 ± 63	380 ± 65	338 ± 73
Scotty	2	522 ± 28	572 ± 36	523 ± 27
	4	454 ± 18	441 ± 28	428 ± 17
	5	395 ± 13	392 ± 12	365 ± 82
	8	337 ±19	334 ± 13	318 ± 24
Stamp	2	685 ± 47	665 ± 36	665 ± 50
	4	497 ± 40	485 ± 31	477 ± 31
	5	429 ± 20	414 ± 16	402 ± 18
	8	341 ± 23	338 ± 19	329 ± 15

At walk (approximately 2m/s) there was a mean time of 585 milliseconds between each R peak, whereas at canter (approximately 8m/s) there was a mean time of 327 ms

between each R value. This demonstrates that, as expected, heart rate increased with increasing exercise intensity.

4.3.2 (ii) Rectal temperature

The rectal temperature increased in all horses following the SET. The mean temperature increase observed throughout the study as a result of the SET was 2.1 ± 0.3 ° C.

4.3.2 (iii) Bodyweight

All horses incurred weight loss as a result of the SET through evaporative losses of sweat and also from faecal loss. The mean weight loss observed as a result of the SET was 5.7 ± 2.4 kg.

4.3.2 (iv) Room temperature

Week of testing	Mean room temperature (°C)
1	23.7
2	14.5
3	21.7

The mean room temperature varied and the temperature in week 2 of testing was between 7 and 9 degrees centigrade lower than weeks 1 and 3.

4.3.3 Saliva

Table 4.4 Free radical scavenging activity of equine saliva

Horse	µmol DPPH scavenged per ml of saliva **Basal**	µmol DPPH scavenged per ml of saliva **Vitamin E treatment**	µmol DPPH scavenged per ml of saliva **no Vitamin E treatment**
Salesman	0.36	0.51	0.62
Scotty	0.38	0.65	0.48
Stamp	0.40	0.55	0.52
Jerome	0.43	0.50	0.49
Barty	0.39	0.52	0.56
Buddy	0.39	0.53	0.47
Mean	**0.39 s.d. 0.02**	**0.54 s.d. 0.05**	**0.52 s.d. 0.05**

A one way ANOVA revealed a significant difference between the groups ($P < 0.01$). A Tukey test showed that there was a significant difference between vitamin E treatment and basal and no vitamin E treatment and basal but no significant difference between vitamin E and no vitamin E treatments.

Free radical scavenging activity of saliva therefore has increased from basal levels but this effect was seen in all horses regardless of vitamin E supplementation.

4.3.4 Sweat Pilot study

The method chosen for use in the study was Whatman 541 filter paper 125mm, held by a plastic cover to the horse's neck. Taping the filter paper directly to the neck was not at all satisfactory as generally it did not stay in place and most of the sweat evaporated during the course of the treadmill test.

Use of the 125mm filter paper consistently collected sufficient sweat for satisfactory TBARS analysis (0.6 – 1.1 g). The 50mm filter paper collected between 0.08 and 0.39 g of sweat, which was insufficient for analysis.

The sweat collected directly from the horse proved to be extremely dirty and even when centrifuged extensively the sample was still heavily contaminated.

The volume used to elute the sweat from the filter paper was 25 ml. This volume was ideal as the eluted samples could be analysed directly, with no further dilution. Volumes of 50ml produced samples that, on occasion, were too dilute and were unable to be detected by the fluorimeter.

4.3.5 Sweat

Table 4.5 TBARS in equine sweat expressed as pmol MDA/g sweat/kg bodyweight

Horse	Pmol/l MDA/g sweat/kg bodyweight **Basal**	Pmol/l MDA/g sweat/kg bodyweight **Vitamin E treatment**	Pmol/l MDA/g sweat/kg bodyweight **No Vitamin E treatment**
Lloyd	7.19	3.57	6.50
Scotty	8.98	5.11	4.73
Stamp	9.19	2.62	10.73
Barty	4.52	4.36	12.86
Buddy	7.60	1.83	6.33
Mean	**7.50 s.d. 1.68**	**3.50 s.d 1.17**	**8.23 s.d 3.05**

A one way ANOVA revealed a significant difference between the groups ($P<0.05$). A Tukey test showed that there was a significant difference between basal and vitamin E treatment and between vitamin E and no vitamin E treatments. Supplementation with vitamin E significantly reduced TBARS in sweat. The level of TBARS in sweat in the no vitamin E treatment was similar to basal values.

4.3.6 Urine

Vitamin C

Table 4.6 vitamin C in equine urine (mg/mmol creatinine)

Horse	mg Vit C/mmol creatinine **Basal**	mg Vit C/mmol creatinine **Vitamin E treatment**	mg Vit C/mmol creatinine **No Vitamin E treatment**
Lloyd	0.76	0.43	0.77
Scotty	0.64	0.79	0.26
Stamp	0.63	0.71	0.31
Jerome	0.51	0.53	0.51
Barty	0.57	0.43	0.76
Buddy	0.63	0.33	0.51
Mean	**0.63 s.d. 0.08**	**0.54 s.d. 0.18**	**0.52 s.d. 0.22**

A one way ANOVA revealed no significant difference between the experimental groups. No change was detected on urinary vitamin C levels, following supplementation with vitamin E.

Urinary TBARS

Table 4.7 Urinary TBARS expressed as pmol MDA/mmol creatinine

Horse	**Pmol MDA/mmol creatinine Basal**	**Pmol MDA/mmol creatinine Vitamin E treatment**	**Pmol MDA/mmol creatinine No Vitamin E treatment**
Lloyd	165.01	155.57	230.39
Scotty	113.62	109.10	117.61
Stamp	228.29	197.26	110.25
Jerome	108.15	278.87	158.09
Barty	144.56	201.14	198.02
Buddy	62.22	131.77	143.26
Mean	**136.98 s.d. 56.83**	**178.95 s.d. 60.77**	**159.60 s.d. 46.81**

A one way ANOVA revealed no significant difference between the experimental groups No change was detected in urinary TBARS levels following supplementation with vitamin E.

4.4 DISCUSSION

4.4.1 PILOT STUDY

The level of TBARS in urine significantly decreased in all horses, after supplementation with Vitamin E (table 4.1). The supplement used was α tocopherol acetate. Much of the acetate is split off in the intestinal wall and the alcohol is reformed and absorbed, thereby allowing the tocopherol to function as a biological antioxidant. The acetate form provides a supplement of exceptional stability and of full biological effectiveness (Roche 1973)

The level of Vitamin E supplementation in this study was 4 mg/kg body weight. This was based on a study by Roneus and Hakkarainen (1986) which showed that to provide adequate Standardbred tissue saturation with α tocopherol, the daily supplement should be 600 – 1800mg. This was equivalent to 1.5 – 4.4 mg/kg BW.

Taking into account the vitamin E contents of the diet, the horses in the current study, when supplemented, were ingesting a mean value of 2.5 g of Vitamin E per day. The current recommended level for Vitamin E in the diet of working horses is 80 mg/kg total diet (NRC 1989).

The vitamin E supplementation appears to have resulted in a marked decrease in TBARS content of the urine. Vitamin E is largely situated in areas of the cell rich in unsaturated fatty acids, such as membranes and blocks free radical attacks on lipids and

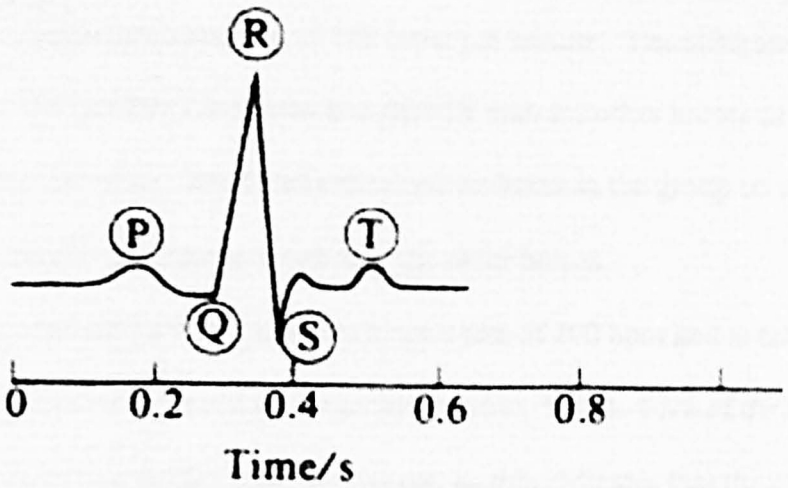
thus the formation of lipid peroxides. The decrease in TBARS could be as a result of decreased lipid peroxidation due to the protective effect of vitamin E.
McMeniman and Hintz (1992) recorded an increase in plasma TBARS in exercised ponies, but no such increase was recorded in exercised ponies supplemented with an additional vitamin E supplement of 100 iu/day (equivalent of 100mg/day).
Avellini *et al* (1999) found that after 70 days of daily training of racehorses along with vitamin E and Selenium supplements, MDA concentration in plasma was reduced and red blood cell resistance to peroxidative stress was increased.

Following these results the study was repeated, with a larger number of horses, using a controlled exercise test. It was decided to decrease the level of Vitamin E supplementation from 4 mg/kg BW to 3 mg/kg BW as the period of supplementation in the second study was increased.

4.4.2 TREADMILL TRIAL

4.4.2 (i) Heart Rate

Electrical impulses generated by the contraction and relaxation of the ventricles of the heart give rise to ECG readings. The heart rate monitor used in this study measures the distance between the R peaks of the ECG reading.



The P wave corresponds to atrial depolarisation prior to contraction, the QRS series shows the ventricular depolarisation. Ventricular repolarisation is shown by the T wave (Pocock and Richards 1999).

RR measurements rely on the R peak of the QRS complex and they measure the time length between the R peaks of each individual heart beat. The slower the heart beat, then the further apart the R peak and the longer time length between the two. At walk (approximately 2m/s) there was a mean time of 585 milliseconds between each R peak, whereas at canter (approximately 8m/s) there was a mean time of 327 ms between each R value. This demonstrates that, as expected, heart rate increased with increasing exercise intensity.

The peak heart rates ranged from 181 – 223 beats per minute and were achieved during the 8 m/s phase of the SET.

Two horses in this study reached heart rates of over 200beats/min. The remaining 5 horses had a mean maximum heart rate of 188 beats per minute. The difference is likely to be due to the fact that Lloyd was less athletic than the other horses as he is generally exercised less often. Buddy was the smallest horse in the group so would have had to work harder at the same speed than the other horses.

V_{200} is the velocity at which a horse achieves a heart rate of 200 bpm and is taken as the maximal aerobic power of the horse (Derman and Noakes, 1994). Five of the horses did not achieve a heart rate of 200 beats per minute, so this indicates that the exercise test was sub-maximal exercise. However, it is difficult to use V_{200} to compare different horses because the maximal heart rate can vary greatly between individuals.

4.4.2 (ii) Rectal temperature

Rectal temperatures were elevated in all horses as a result of the SET, however, this increase was not indicative of fatigue due to hyperthermia. A fan was positioned in front of the horses, moving air over the body as the horse moves forward. In addition, the horses underwent a period of cool down whilst on the treadmill and were then walked in hand until they were dry.

4.4.2 (iii) Bodyweight

All horses incurred weight loss as a result of the SET. Cutaneous evaporation represents the primary mechanism for heat dissipation in the horse (Kingston *et al* 1997). Equine sweat is hypertonic in relation to plasma (McCutcheon *et al* 1998) with

sodium, chloride and potassium representing the major losses. All horses in this study received electrolyte supplementation following the SET to combat fluid and electrolyte imbalances.

4.4.2 (iv) Environmental Temperature

Environmental conditions could not be controlled during this study. The mean room temperature varied and the temperature in week 2 of testing was between 7 and 9 degrees centigrade lower than weeks 1 and 3.

In cooler conditions heat loss through evaporation may decrease, thus reducing water losses and enhancing performance. However, the weight losses incurred in week 2 were not significantly different from weeks 1 and 3 and the heart rate data did not indicate that the horses were working at a different intensity in this particular week.

4.4.3 Saliva

Saliva was collected from six horses out of seven as one horse would not tolerate the saliva collection bit. The results show a significant increase in the free radical scavenging activity of saliva after the control week, which was week one of the trial. The free radical scavenging activity of the saliva however has increased regardless of whether the horses were receiving a vitamin E supplement.

The free radical scavenging activity of saliva gives an indication of the antioxidant capacity of the saliva and therefore, blood. The indication is that the antioxidants in the system have increased to the same extent in vitamin E and no vitamin E treatments, so it

is likely that it is not the vitamin E which is causing this effect. One possibility is that the antioxidant defence systems have increased as a result of exercise. Exercise training has been reported to promote an increase in skeletal muscle antioxidant enzyme activity (Dekkers *et al* 1996).

White *et al* (2000) measured antioxidant capacity in plasma (PAOC) and found an increase in plasma antioxidant capacity as a consequence of exercise. This was attributed, in part, to a compensatory response to the increased amount of pro-oxidant species.

However, this does not explain the significant difference between the control week and the other two weeks. It could be suggested that it is a training effect, but these horses had performed the same exercise test 3 times a week for 3 weeks prior to this trial, so it is unlikely that this is the case.

The explanation may be due to the fact that the horses were being kept at grass. The horses are normally stabled and are turned out to grass for half a day and then stabled again at night. However, during the current study the horses were turned out at grass for 24 hours a day, with the exception of the night before the treadmill test. The horses had been turned out to grass only 2 days before the start of this trial, so during the control week the horses had been ingesting high levels of grass for only a few days. However, in the second week of testing on days 15, 17 and 19, the horses had been ingesting grass for nearly 3 weeks. This could provide the explanation for the increased antioxidant levels in the saliva.

Table 4.8 The content (mg/kg) of fat soluble vitamins in grass. Taken from Holmes (1989) Grass: Its production and Utilisation page 121.

Grass	**β- carotene**	**Vitamin E as total tocopherols**
Fresh	9 – 127	105 – 166
Hay	3.9 – 18	200
Silage	8 – 13	38 – 470

As well as the vitamin E supplement, the horses were ingesting significant levels of vitamin E from fresh grass. The high levels of vitamin E from the grass may have been sufficient to mask the effect of the vitamin E supplement. β carotene is also present in high quantities in grass. It exerts antioxidant functions such as quenching of singlet oxygen and peroxyl or alkoxyl radicals (Sies and Stahl 1995). It is also, like vitamin E, a lipid soluble antioxidant. It is likely therefore that the ingestion of high levels of vitamin E and β carotene may explain the increased antioxidant capacity of the saliva. There is a significant difference in the free radical scavenging activity of the saliva between the vitamin E and basal group but no difference between vitamin E and no vitamin E treatments. As vitamin E is a fat soluble vitamin it is possible that the vitamin E has remained stored in the adipose tissue and the liver, and the antioxidant status of the animal remains elevated once the vitamin E supplement is removed.

Where α tocopherol and β carotene are present in the same system α tocopherol protects β carotene from oxidation (Palozza and Krinsky 1992). It was suggested that this protection is dose dependent and occurs only at sufficiently high concentrations of α tocopherol. The hypothesis of α tocopherol protection of β carotene was demonstrated in a study by Palozza and Krinsky (1995) where there was an increase of α tocopherol consumption induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) in the presence of a combination of the 2 antioxidants. Kozachenko *et al* 2000 found that α tocopherol inhibited β carotene oxidation even when the α tocopherol concentration was 0.56×10^{-5} M.

The interactions between α tocopherol and β carotene may have had a bearing on the results obtained in the present study. If α tocopherol, from either the grass or the supplement, has exerted a protective effect on β carotene then β carotene would not be oxidised by free radicals and the antioxidant capacity of the animal would remain high. This may explain why there was no difference between vitamin E and no vitamin E treatments, but increased levels of antioxidants in the system.

4.4.4 Sweat TBARS

Results were obtained for five horses out of the seven as insufficient sweat was collected for two of the horses. The measurement of TBARS in equine sweat is a novel technique and the results demonstrate a significant decrease in sweat TBARS concentration following supplementation with α tocopherol. In addition, the levels of

TBARS increased again once the supplement had been removed from the diet. These results suggest that despite high intakes of vitamin E and β carotene from grass, α tocopherol supplementation has affected lipid peroxidation.

This could be due to the α tocopherol from the supplement decreasing lipid peroxidation in the lipid membrane, consistent with the results from the pilot study. Sweat is derived from ICF and ECF so it could be argued that TBARS in sweat is a reflection of TBARS, or MDA in the circulation. Another explanation for decreased TBARS in sweat could be that one of the routes of excretion of vitamin E is through the skin. The horses were receiving high levels of vitamin E in grass and it is possible that the vitamin E administered as a supplement was excreted through the usual routes, one being the skin. It is possible that vitamin E was collected in the sweat as well. However, MDA is an end product of lipid peroxidation and would not be affected by the presence of an antioxidant so this should not have affected the measurements.

It has been found that the skin possesses an efficient and unique antioxidant activity (Kohen and Gati 2000). It was found that following exposure to oxidative stress the release of low molecular weight antioxidants (LMWA) from the skin was significantly enhanced (Kohen and Gati 2000).

During exercise there is an increase in the metabolic requirement of a number of tissues. In response to this increased demand there is a primary increase in blood flow to exercising muscles and a relative decrease in flow to skin, resting muscle, the kidneys and the splanchnic region in proportion to exercise intensity (McConaghy 1994). In addition to the response to exercise, these trials were carried out in the summer and

although the horses were cooled with fans during the exercise test they were still exercising in heat, which may suggest that skin blood flow is increased. However, if blood flow to the skin is increased then there must be a reduction in blood flow to working muscle, which would then potentially reduce metabolic performance. Skin blood flow changes have not been measured in the horse but the thermoregulatory responses of horses are similar to those of humans. In humans blood flow to working muscles is not compromised during exercise and heat stress, so skin blood flow must be reduced (McConaghy 1994).

This reduction in blood flow could lead to transient tissue hypoxia. This has been shown to lead to an increase in hydrogen ions, which can react with superoxide anions to ultimately produce additional reactive oxygen species (Jenkins 1993). Tissue hypoxia can also lead to the freeing of transition metals such as iron and copper from their normal transporters. These free metals can further catalyse free radical reactions (Kanter 1998).

It is therefore possible that the skin is indeed exposed to oxidative stress during exercise. If the skin has its own response to oxidative stress by releasing LMWA then this will reduce MDA production. However, it still does not explain the marked decrease in sweat TBARS in the horses when treated with alpha tocopherol. It is therefore likely that the vitamin E supplement has decreased lipid peroxidation in these exercising horses.

McMeniman *et al* (1992) investigated the effect of vitamin E status on lipid peroxidation in exercised horses. Overall they found that exercise caused an increase in

plasma TBARS and that vitamin E supplementation was consistent with the reduction in plasma TBARS levels.

Petersson *et al* (1991) investigated the effect of vitamin E on the membrane integrity of eight Standardbred horses during submaximal exercise. The results indicated that vitamin E supplementation caused a rise in vitamin E status and a decrease in indicators of lipid peroxidation (TBARS and expired pentane).

4.4.5 (i) Urinary TBARS

Urinary TBARS showed no significant response to vitamin E supplementation. Although not significant, the mean values for urinary TBARS in the horses treated with vitamin E increased. *In vitro* studies have demonstrated that under certain conditions vitamin E can act as a pro-oxidant. Kontush *et al* (2001) investigated the antioxidant and pro-oxidant activity of α tocopherol in human plasma and low density lipoprotein. A pro-oxidant effect of α tocopherol was found in isolated LDL and mild oxidative conditions in plasma, where there were also low levels of co-oxidants such as ascorbic acid, which is capable of regenerating α tocopherol. The authors concluded that under conditions of mild oxidation where co-oxidants are exhausted α tocopherol may evolve into a pro-oxidant.

However, is this likely? The horses were not performing maximal exercise, therefore it could be said that mild oxidative conditions would apply.

Vitamin C is water soluble and is considered to be the most important antioxidant in extracellular fluids (Sies and Stahl 1995). Ascorbic acid reduces the tocopheroxyl radical and thereby restores the radical-scavenging activity of tocopherol.

β carotene oxidation has been seen to be inhibited by the presence of ascorbic acid (Kozachenko *et al* 2000). It is possible that the high intake of vitamin E and β carotene exhausted vitamin C which could lead to vitamin E acting as a pro-oxidant.

Snow and Harris (1989) state that ascorbic acid concentrations are lower in horses than in other species and suggest that supplementation may have a place in the exercising horse. Low levels of vitamin C combined with high intakes of vitamin E and β carotene may have led to increases in the α tocopherol radical and perhaps even the β carotene radical, which would exert pro oxidant effects.

However, the antioxidant capacity of saliva increased and TBARS in sweat decreased, so overall a pro-oxidant effect is unlikely.

A study by Vannucchi *et al* (1997) investigated the effect of three different dietary levels of vitamin E (normal, 20 times higher than normal and deficient) on plasma and liver lipid peroxidation in rats, assayed by determination of TBARS. Plasma TBARS did not differ between the groups, although hepatic TBARS production was higher in the deficient group. Although plasma TBARS did not differ, they were none the less inversely correlated with liver concentration of Vitamin E. Plasma vitamin E was

higher in the supplemented group and a significantly higher accumulation of α tocopherol was observed in the liver of the supplemented group, which at least demonstrates that extra vitamin E is retained in the plasma and liver and is not excreted.

A study by Kuwabara *et al* (1995) examined lipid peroxide levels in the sera of hotbred (Thoroughbred) horses, determined by TBARS in serum, and compared with lipid peroxide levels in coldbred (cross bred) horses. There were significantly lower levels of TBARS in the sera of coldbred horses. So Thoroughbreds were shown to have naturally higher levels of TBARS, regardless of exercise (TBARS in sera were measured in Thoroughbred foals, breeding stock and racehorses and no difference between them was noted). It was also found that hotbred horses had significantly higher superoxide –scavenging abilities (in sera) than coldbreds. In addition, racing hotbreds had significantly higher superoxide scavenging abilities than the other classes of hotbreds (breeding and foals).

This suggests that the high superoxide scavenging ability of sera from racing horses may contribute to keeping their lipid peroxide levels as low as those of foals and horses for breeding purposes (Kuwabara *et al* 1995).

All the horses used in the present study were fit Thoroughbreds similar to those used in Kuwabara's study. It is possible that an already efficient antioxidant system means that supplements with antioxidants are not necessary, which is why there was no significant response in urinary TBARS. However, the confounding factor is that while TBARS in urine remains unchanged, TBARS in sweat decreased. It may be possible that because saliva and sweat are derived from blood they give an indication of what is occurring

during exercise, but urine was collected 0.5 – 2 hours after exercise, and this may explain the difference. As demonstrated by the heart rate, the exercise test was not working the horses anaerobically so it is possible that lipid peroxidation was not greatly affected. It is possible that TBARS in sweat is a more sensitive indicator than TBARS in urine.

One interesting observation is that there is a fairly strong negative correlation between urinary TBARS and free radical scavenging activity of saliva ($r = -0.64$). This is not significant but nevertheless there is some evidence of association. This means that where there is an increase in urinary TBARS, which demonstrates increased lipid peroxidation there is a decrease in the free radical scavenging activity of saliva, and therefore blood.

A decrease in urinary TBARS following vitamin E supplementation was observed in the pilot study. This difference is likely to be due in part to the season. The horses in the current study were ingesting vitamin E from pasture as well as from their normal rations. The pilot study was conducted in November/December when the horses were stabled, had no access to pasture and were eating conserved forage. This could lead to the conclusion that vitamin E supplements are useful during the winter months when horses have limited access to pasture and perhaps are not useful in the summer when horses are consuming good quality fresh pasture. Secondly, the horses in the current study received a vitamin E supplement of 3mg/kg BW and the horses in the pilot study received 4 mg/kgBW. However the horses in the current study received the supplement

for longer, plus the extra vitamin E from grass, so overall the horses in this study probably received more vitamin E. This again may suggest a pro-oxidant effect but this is not supported by the sweat TBARS data.

4.4.5 (ii) Urinary ascorbic acid

Urinary ascorbic acid levels showed no significant response to vitamin E supplementation.

Ascorbic acid is a water-soluble vitamin synthesised in the horse from glucose. It acts as a water- soluble antioxidant `and traps peroxide radicals (White *et al* 2001). It acts mainly in plasma, where it has been found to contribute up to 24% of the total radical trapping capacity in humans (Wayner, 1987).

Ascorbic acid regenerates α-tocopherol from the alpha-tocopheryl radical by electron donation. This reaction has been shown to take place on or near the outer membrane surface, as ascorbate and α-tocopherol cannot interact within one medium, due to their hydrophilic and hydrophobic properties, respectively (Niki, 1999).

It was expected that as vitamin C was used to regenerate α-tocopherol, the level of vitamin C excreted in urine would decrease with vitamin E supplementation, however this was not demonstrated in this study.

The method used for determination of ascorbic acid was titration with 2,6 dichlorophenolindophenol in acid solution. On titration with ascorbic acid the dye is reduced to colourless leucobase, the ascorbic acid being oxidised to dehydroascorbic acid. If a mixture of dehydroascorbic acid and ascorbic acid is present only the latter

reacts with the dye, although both are biologically active. In urine only ascorbic acid appears to be present and analysis needs to be determined on fresh specimens so that oxidation of ascorbic acid does not occur. The addition of acetic acid in the proportion of 1 part acid to 9 parts of urine will delay oxidation of ascorbic acid for several hours. However, other compounds which decolourise the dye are also present in urine, although they react more slowly than ascorbic acid in acid solution. Nevertheless it is possible that this test is not sensitive enough to detect small changes in ascorbate levels in urine.

High pressure liquid chromatography is the best method for analysis of vitamins, but unfortunately HPLC was not available for the present studies.

In a study by White *et al* (2001), antioxidant capacity, endogenous and exogenous ascorbate concentration, total antioxidant reactivity and TBARS were measured in plasma of Thoroughbred race horses both with and without an ascorbate supplement. Plasma ascorbate concentrations in horses treated and untreated with this antioxidant did not change as a result of exercise. This was in accordance with other studies (Snow, 1990; McMeniman and Hintz, 1992) where plasma ascorbate did not increase as a result of work. However, White *et al* (2001) suggest that there could be variations in the ascorbate/dehydroascorbate ratio.

L – ascorbic acid is oxidised to monodehydrascorbate, an ascorbate radical. Loss of another electron, through reaction with a free radical yields dehydroascorbate (Bender, 1995). Dehydroascorbate is biologically active. It is readily oxidised to form 2, 3-

diketogulonic acid, which has no biological activity and represents a loss of vitamin C (Brody 1999).

Ascorbic acid can be regenerated from dehydroascorbate by dehydroascorbate reductase which utilises glutathione, the levels of which are maintained by glutathione reductase (Brody 1999).

It is possible that as ascorbic acid regenerated vitamin E it is oxidised to monodehydroascorbate and then further to dehydroascorbate but is then regenerated and so is not lost from the system.

In addition, ascorbic acid is only excreted once blood plasma exceeds its normal threshold of approximately 1.4mg/100ml (McDowell 1989). If plasma levels do not change, as demonstrated in the study by White *et al* (2001), then there will be no change in urinary excretion.

CHAPTER 5

GENERAL DISCUSSION

The aim of the present series of studies was to investigate the potential usefulness of non-invasive measures of oxidative stress in the horse. Urinary TBARS, sweat TBARS, and free radical scavenging activity of saliva were used to measure oxidative stress and urinary NAG activity was used as an indicator of renal insult. In addition, ^{1}H NMR spectroscopy was used to examine equine urine, particularly to establish any age-related change.

The results are summarised below:

- Urinary NAG activity could not be easily measured in the horse.
- ^{1}H NMR spectra were obtained and senior horses showed increased urinary excretion of aromatic amino acids compared to younger horses. Increased total proteinuria in senior horses, as measured by the biuret assay was also noted.
- Urinary TBARS in horses could be measured using a method adapted from Yagi (1976) and were significantly decreased in horses following supplementation with antioxidants in the form of dandelion and milk thistle. However, urinary TBARS in horses performing a sub-maximal exercise test on a treadmill were unaffected by vitamin E treatment. Urinary TBARS were seen to increase in older horses compared to younger horses.

- TBARS in equine sweat could be measured using a method adapted from Yagi (1976). TBARS in sweat were significantly decreased in horses performing a sub-maximal exercise test on a treadmill, following vitamin E supplementation.
- Free radical scavenging activity of equine saliva could be measured using a method adapted from Atsumi *et al* (1999).

5.1 Urinary NAG Activity

NAG activity appeared to be low in the urine of horses and was not easily measured. This was thought possibly to be due to large volumes of urine produced by the horse, which effectively 'diluted' the enzyme beyond detectable limits. Sato *et al* (1997) reported that urinary NAG activity in healthy cattle was low compared to values for other animals. Cattle lose a lot of water through faeces (75-85%) so it is possible that higher urinary flow rates in horses means that NAG activity is lower still in horses.

Two horses had evidence of urinary NAG activity, once urine had been concentrated using Microcon filters. The history of these two horses showed that one had received high levels of phenylbutazone in the past and the other was being investigated for selenium deficiency. This suggests that NAG activity cannot be easily measured in the urine of healthy horses, but may be detected in urine samples from diseased horses. This may suggest that urinary NAG may be a positive screening tool, which can be used to detect renal insult in horses that have received high levels of phenylbutazone, for example. Sato *et al* (1999) measured urinary NAG activity in the urine of cows with renal parenchymal lesions of tubular cells and found elevated NAG activities compared with those of healthy cattle.

Concentrations of blood urea nitrogen (BUN) and serum creatinine were low in all cows in the study indicating that changes in NAG activity in urine may yield information about kidney damage before an increase in BUN or serum creatinine concentration, which occurs after 50% of all nephrons are injured.

Although for the purpose of the current studies urinary NAG activity did not yield information regarding renal insult following exercise or during ageing, it may be potentially clinically useful.

5.2 TBARS

The use of the TBA test as evidence for *in vivo* peroxidation is limited by its lack of specificity (Gutteridge and Tickner 1978). Despite these criticisms, the method is one of the most useful and commonly used measurements as it is an easy and quick assay for the assessment of lipid peroxidation.

HPLC – based TBA assays can now be used to increase specificity, but were not available for the present studies. Lykkesfeldt (2001) quantified MDA in plasma, erythrocytes and liver homogenates from guinea pigs using HPLC with fluorescence detection, which quantifies only the actual MDA – TBA adduct. They compared this with the original and less sensitive spectrophotometric method which measures absorbance of several species. They observed substantial overestimations in samples measured spectrophotometrically compared with HPLC and suggest that actual concentrations reported from spectrophotometric detection should be regarded as rough estimates.

Hoving *et al* 1992 analysed the MDA – TBA adduct in plasma lipid extracts using spectrophotometry, fluorometry and HPLC with fluorometric detection. The authors

found good agreement between the three methods with higher values also being noted for the spectrophotometric method.

The use of HPLC would increase the specificity of the fluorometric assay, however for the purpose of the current studies the TBA assay was used as a comparative index of lipid peroxidation.

5.2.1 Use of urine vs plasma or serum

Biomarkers reflect changes in biological systems that are related to an exposure to or effects from a potentially toxic compound.

De Zwart (1998) lists the following characteristics of an ideal biomarker:

1. high specificity for the effect of interest
2. reflection of an early interest
3. easy and inexpensive analysis
4. medium available by non-invasive sampling techniques
5. low background level of the biomarker in the medium of interest
6. a well-established relationship between the response of the biomarker and exposure
7. a well-established relationship between the response of the biomarker and the induced damage

In this context, the relationship between lipid peroxidation and MDA has been well established. The published data regarding free radical production in horses is generally concerned with concentrations of MDA in muscle and blood samples. However, breakdown products of lipid peroxidation are generally removed from the body into urine, or into inhaled air, so monitoring urinary excretion of breakdown

products of peroxidized lipids is a useful way of identifying oxidative damage (de Zwart 1998). Determination of TBARS in urine has been used to establish the involvement of lipid peroxidation in humans (Siciarz *et al* 2001, Mikami *et al* 2000, Jenkinson *et al* 1999). For example, diabetes mellitus is associated with increased free radical formation (Baynes 1991) and MDA, measured as TBARS, is the most extensively studied marker in diabetes related lipid peroxidation and significantly higher levels of MDA in urine were found in humans with both type 1 and type 2 diabetes (de Zwart 1998).

Ekstrom *et al* (1986) reported a significant increase in urinary MDA excretion of phenobarbital – pretreated rats exposed to chloroform or CCl_4.

In terms of the specificity of the assay used in the current studies the assay is non-specific for any one class of peroxidation product but is a valid measure of overall peroxidative changes during ageing and exercise. The use of a fluorometric technique, although not as specific as HPLC means that the analysis is straightforward and inexpensive.

Collection of samples using non-invasive methods has the advantage over venous or capillary sampling as it is less stressful, sparing the uncomfortable experience of repeated venepunctures. In the horse apprehension has been shown to cause splenic contraction, elevating blood haemoglobin concentration by approximately 50% (Hornicke *et al* 1977), causing variation in blood samples.

Analysing urine in horses is desirable because it is non-invasive, allows for a greater number of samples to be collected and is easy to process. The disadvantages are variable composition, although standardisation with creatinine can overcome this to some extent, it is not as well established as using blood, and lack of control over

time of collection of the sample can be problematic. It can also be very difficult to obtain urine samples from horses. The difficulty arises where only a small population of horses is available, (for example, those used in the studies described in chapter 4) and it is essential to obtain a urine sample from each horse. Horses can be sensitive regarding urine collection and extremely stubborn which can make the process at best frustrating and at worst invalidate the study. Nevertheless, it is important to try to develop non-invasive methods and such difficulties are not insurmountable.

5.2.2 Urinary TBARS in horses

Urinary TBARS were measured in the urine of horses using a fluorometric method adapted from Yagi (1976). Urinary TBARS appeared to vary quite widely between individuals, but within individual values were fairly consistent. The results in Section 2.3.2 did not demonstrate an increase in urinary TBARS in exercising horses compared to non-exercising horses. However, exercise has been shown to increase the level of lipid peroxidation, (Sumida *et al* 1989; Witt *et al* 1992; Marlin *et al* 2002) and Kosugi *et al* (1994) found that increased physical activity in healthy humans increased the excretion of urinary TBARS. However, the increase in lipid peroxidation is related to the intensity of the exercise and these horses were not working particularly hard. One problem in equine science is that there is no standard classification for work load. Measurement of heart rate will obviously define whether the work is aerobic or anaerobic but there is no simple physiological way of measuring work. For example, is it harder to perform a gallop around a steeplechase or to perform a Grand Prix dressage test? When calculating energy

requirements the NRC equation used is a function of bodyweight and work load. Although work load can be regarded as a function of bodyweight, intensity and time spent, in practice where horses perform such a wide range of disciplines this is difficult to apply.

Urinary TBARS decreased significantly in the urine of horses supplemented with dandelion and milk thistle. These herbs contain significant amounts of antioxidants in the form of flavonoids and antioxidant vitamins and minerals. This was a positive result because not only did it demonstrate that urinary TBARS respond to antioxidant supplementation in horses but also that these two herbs are useful antioxidants. There are a vast number of herbal products for horses on the market and very little scientific data to demonstrate their efficacy. It is a concern that herbs are often regarded as 'natural' and therefore 'safer' than synthetic drugs. Any herbs that have a physiological effect on the horse does so due to an active pharmacological ingredient and a number of herbs carry the risk of toxicity. Research into the effects of herbs on horses *in vivo* is limited and further studies are required.

Urinary TBARS were also seen to increase with age, which is consistent with increased lipid peroxidation in senior horses. However, urinary TBARS did not respond to supplementation with α-tocopherol during the controlled exercise test. There are a number of reasons why this might have been the case, which are discussed further but a factor could be the timing of the urine collection. Mikami *et al* (2000) found that urinary TBARS excretion in humans following exhaustive exercise decreased during the first 60 minutes of the recovery period and thereafter significantly increased during the latter half of the recovery period. Plasma TBARS

measured in horses immediately after intense exercise over jumps was not significantly different to pre exercise values but was seen to significantly decrease after 24 hours of rest (Balogh *et al* 2001). Maughan *et al* (1989) found that serum TBARS did not return to pre-exercise levels until 72 hours after exercise. Urine samples in the current study were collected mainly within 1-2 hours of exercise. It may be the case that if urinary TBARS are to be used as an indicator of oxidative stress during exercise, then samples should be collected post exercise and then again 24 hours later. It may also be the case that urinary TBARS are not the best indicator of oxidative stress during exercise. It appears from the results from the senior trial and from the herb trial that urinary TBARS are useful when looking at changes in lipid peroxidation over a period of time. It may be more appropriate to measure urinary TBARS pre and post trial, to monitor how increased exercise has affected horses over a period of weeks rather than individual exercise bouts. Other body fluids such as sweat or saliva may provide more 'immediate' information regarding oxidative stress during an exercise bout.

5.3 EXERCISE

5.3.1 Treadmill trials

Treadmills are extremely useful as the physiological responses to exercise can be closely monitored and the speed, duration and intensity of work can be controlled. However, treadmill exercise cannot duplicate the effects of air movement, ground surface, and the impact of a rider. Horses on a treadmill have no momentum as the treadmill itself provides the driving force, so the actual work performed by horses

during treadmill exercise is quantitatively different from track work (Rose and Hodgson 1994).

Inclining the treadmill can increase the amount of mechanical work performed in order to match the physiological and locomotor responses to track conditions (Barrey *et al* 1993). Barrey *et al* (1993) concluded that the optimal slope for reproducing the same heart rate response as horses being ridden ranged from 3.0 to 3.7^{0}. The incline used in the current studies was 6^{0}. This means that a higher overall workload can be achieved at lower speeds.

Acclimatisation to exercise on a high speed treadmill is necessary and the time a horse needs to acclimatise can range from a few minutes (Buchner *et al* 1994) to three to five sessions (Seeherman *et al* 1990). All horses in the current trial had worked on the treadmill many times and were familiar with the exercise test and equipment used. It has been noted that horses get excited prior to a treadmill test and this can sometimes make a test less reliable, for example, heart rate can be rapidly elevated (Sloet *et al* 1999).

5.3.2 Vitamin E supplementation

The aim of this series of experiments was to use a variety of techniques to measure oxidative stress in exercising horses and to investigate the effect of an antioxidant supplement.

Vitamin E was given to working horses as a supplement and, initially urinary TBARS were used to establish whether or not this antioxidant would reduce oxidative stress during exercise. A pilot study was carried out to determine whether vitamin E would be the most appropriate antioxidant where 4 horses were

supplemented with α tocopherol at a level of 4mg/kg BW for 1 week. The results showed a significant decrease in the level of urinary TBARS following supplementation.

Following these promising results a further study was carried out using a controlled exercise test and a cross over design to investigate the effect of α tocopherol supplementation on oxidative stress in the horse, as assessed by urinary TBARS, sweat TBARS, free radical scavenging activity of saliva and urinary ascorbic acid. In this trial there was no significant difference in the levels of urinary TBARS following supplementation with α tocopherol.

There are several reasons why this might have been the case. The pilot study was carried out in the winter months and the horses were eating conserved forage, as no fresh grass was available. The treadmill trial was carried out in the summer when the horses were being kept at grass during the day. Fresh grass contains high levels of vitamin E, so in addition to the α tocopherol supplement (fed at a level of 3mg/kg BW) the horses were receiving additional vitamin E from fresh grass. The vitamin E from the concentrate part of the diet would have been similar in both trials as the horses were receiving similar commercial feeds (for details of feed see appendices 4 and 5).

Losses of vitamin E during haymaking can be as high as 90% (McDonald *et al* 1995) so the horses eating hay in the winter months would be receiving significantly less vitamin E in the diet. It may be the case that the horses eating grass in the summer were ingesting more than sufficient vitamin E in the diet and the supplement was simply excreted. A comparison of urinary TBARS values from the

two trials shows that the urinary TBARS levels of the horses in the treadmill trial, pre-treatment (the control values) were much lower than those in the pilot study which is consistent with decreased lipid peroxidation. This may also be due to differences in body condition between the two populations of horses. The horses in the pilot study were carrying more fat than the horses in the treadmill trial. Data from experiment 2.2.2 indicated that fatter horses had higher levels of urinary TBARS compared to horses of a lower body condition score (although these urine samples were not standardised with creatinine). It may be the case that a similar trend is exhibited here.

Results from experiment 2.2.4 where horses were supplemented with antioxidants in the form of dandelion and milk thistle demonstrated a significant decrease in the level of urinary TBARS. This trial was also carried out in November/December, again suggesting that supplementation of antioxidants during the winter months may be beneficial.

The level of vitamin E supplementation was reduced from 4 mg/kg BW in the pilot study, to 3 mg/kg BW (refer to appendices 4 and 5). However the horses in the pilot study received the supplement for 1 week, while in the second trial the horses received the supplement for 2 weeks. However, it may be the case that the level of 3mg/kg BW was not effective, although McMeniman and Hintz (1992) recorded a decrease in plasma TBARS in exercised ponies supplemented with an additional vitamin E supplement of 100 iu/day (equivalent of 100mg/day).

Another explanation could relate to the fact that the treadmill trial involved a cross over design. 4 horses out of 7 received the vitamin E supplement for 2 weeks and were then swapped over following an exercise test. It may be that as vitamin E is a

fat- soluble vitamin the 4 horses tested during the 'no vitamin E' treatment, following vitamin E supplementation, may have still demonstrated elevated vitamin E levels and hence no effect was seen. The way to avoid this problem would be to include a washout period in-between treatments. This would be the ideal solution but limitations of time meant that this could not be implemented in this trial. However, much less vitamin E is retained in the body than other fat soluble vitamins such as vitamin A. Kinetic studies in humans have shown that a maximal binding capacity for α tocopherol may exist (~ 50mg) within the plasma, thereby leading to faecal excretion of excess vitamin E (Traber 1999). Vitamin E recovered in faeces from a test dose has been found to be in the range of 65-80% in man, rabbit and hen (Roche 1973) and the storage of vitamin E in the body appears to be limited.

This would suggest that the horses out at grass in the summer, ingesting high levels of vitamin E from fresh grass, excreted the extra supplement. This could lead to the recommendation that vitamin E supplements may be useful during the winter months or for horses who have limited access to fresh pasture, such as racehorses but this hypothesis would need to be tested further.

The level of exercise in the pilot study could not be quantified as the horses used were working in the school. The second trial as seen by the heart rate data can be classified as sub-maximal exercise. It may be that the exercise test was not intense enough. The intensity and duration of the exercise appears to influence the appearance of lipid peroxidation, in that an increase is more likely to be seen at VO_2 max (Witt *et al* 1992). It was anticipated that the exercise would be anaerobic using this treadmill test. Horses on previous trials using this particular test achieved heart rates of 200 bpm and over, which is taken as the aerobic threshold. However, these

previous trials took place in November/December when the horses had been working for only 2 or 3 months following their summer break. The current study took place in the summer when the horses had experienced nearly a full year of work and were comparatively much fitter.

5.3.3 Sweat TBARS

TBARS were measured in equine sweat and there was a significant decrease in sweat TBARS following supplementation with vitamin E. Additionally, the levels of TBARS in sweat increased back up to control or basal levels once the vitamin E supplement had been removed from the diet. In contradiction to urinary TBARS this suggests that despite high intakes of vitamin E from grass, the α tocopherol supplement affected sweat TBARS.

The measurement of TBARS in sweat is a novel technique. The assay was the same as for urine, using the method adapted from Yagi (1976). The sweat was eluted from filter paper using 25 ml of distilled water and there was no need to further dilute the samples. Sweat TBARS was expressed as MDA/g sweat/kg bodyweight. The reason for including the bodyweight was that sweat production is related to body size and this was an attempt to try and standardise the results. Variations in composition in urine can be overcome by expressing urinary TBARS as a ratio of MDA: urinary creatinine. The daily excretion of creatinine in individuals is fairly constant on a daily basis as it relates to muscle mass, but there is nothing comparable in equine sweat. One possibility could be the protein latherin, which is found in high concentrations in equine sweat and has a surfactant-like action, promoting spreading and evaporation of sweat (McConaghy 1994).

The results demonstrate that there were thiobarbituric acid reactive substances in equine sweat and that these substances were affected by vitamin E. To confirm what these substances actually are a more specific assay should be employed such as HPLC and mass spectrometry to try to identify a TBA-MDA adduct.

Also, it would be helpful to identify the origin(s) of the TBARS. The method of collection means that it is possible that TBARS are originating from the skin. Alternatively, as sweat is derived from ECF and ICF the TBARS in sweat could more closely reflect events occurring in the plasma and so it is important to establish the relationship between sweat and plasma TBARS. In the current study no significant correlation was found between sweat TBARS and urinary TBARS.

The use of sweat TBARS appears to be a promising non-invasive biomarker of lipid peroxidation, but further work would need to be done to establish the reliability and validity of this assay.

5.3.4 Free radical Scavenging activity of equine saliva

Free radical scavenging activity of equine saliva was measured. The activity was measured in terms of the rate of decrease in the absorbance at 517 nm in a 40 % ethanol DPPH solution. This was based on a study performed by Atsumi *et al* (1999) where total free radical scavenging activity in the low-molecular weight non-enzymic fraction of human whole saliva was measured. The mean DPPH activities of saliva and serum showed a significant linear relationship and physical exercise significantly decreased activity, in humans.

The concept of a test that reflects total antioxidant capacity to use alongside other measures of free radical damage is an attractive one. Low total antioxidant capacity

could be indicative of either oxidative stress or an increased susceptibility to oxidative damage (Koracevic 2001). Total antioxidant capacity is different to free radical scavenging activity as total antioxidant capacity suggests that all antioxidants are being assessed. In saliva, it is likely to be the low molecular weight chain breaking antioxidants, such as vitamins and minerals, which will scavenge the free radicals rather than antioxidant enzymes. Atsumi *et al* 1999 found that heating or freeze-thawing did not influence the DPPH scavenging activity of whole saliva, indicating that the main source for scavenging the DPPH radicals is the non-enzymic fraction.

The method used to collect saliva was by use of a specially designed bit connected to a vacuum pump. In effect, saliva was sucked out of the horse's mouth during the exercise test. Other studies where equine saliva has been collected have used swabs (Linder *et al* 2000). Studies in humans have found that some salivary biomarkers were subject to interference effects caused by cotton materials used to absorb saliva during sample collection and that for some biomarkers this collection method can be a significant source of unsystematic error (Shirtcliff *et al* 2001). For this reason a method of collection was devised to collect the saliva directly. Saliva was successfully collected using this method. In the first instance the saliva was heavily contaminated with hay, so during subsequent trials hay and concentrates were removed prior to the exercise test. Even so it was still difficult to totally eliminate the effect of nutrition. Another problem was that there was a large variation in flow rates. Some individuals produced a small quantity of saliva (approximately 1ml) while others produced approximately 5 ml. Salivary flow rate in humans has been shown to have a significant influence on salivary composition (Chicharro *et al*

1998). A study performed by Eckersall *et al* (1985) collected whole saliva from six horses over a period of 5 weeks, using a sherbet lemon sweet to stimulate salivation. There was considerable variation in the concentration of the analytes both between horses and between different days in the same horse, the most variable constituent being sodium. In the current study the coefficient of variation for the values obtained between the 6 horses in the study were reasonable, being 0.05, 0.03 and 0.10 for weeks 1, 2 and 3 respectively. However, the coefficient of variation within individuals was quite high, ranging from 0.058 – 0.325. Again, it would be desirable to find an analyte which could be used to standardise the samples.

In the study by Atsumi *et al* (1999) the mean DPPH activities of saliva and serum in humans, showed a significant linear relationship. This relationship has not been established in the horse and is necessary to validate the use of this assay in horses. The aim was to investigate the effect of vitamin E on the free radical scavenging activity of equine saliva. Treatment with vitamin E did not affect DPPH activity, but a significant increase in the free radical scavenging activity of saliva after the control week, which was week one of the trial, irrespective of the vitamin E supplement was noted. This was thought to be due to the increased ingestion of grass, which contains high levels of antioxidants (Holmes 1989). Although the aim was to establish the effect of a vitamin E supplement, it is positive that the free radical scavenging activity of the saliva reflected this increased intake of antioxidants and further work to validate this assay for use in the horse is required.

5.3.5 Antioxidants

Ascorbic acid regenerates α-tocopherol from the alpha-tocopheryl radical by electron donation. Urinary ascorbic acid was also measured to determine any effects of vitamin E supplementation but no significant difference between treatments was noted. This was thought to be due to the fact that ascorbic acid is only excreted in urine once blood plasma exceeds its normal threshold of approximately 1.4mg/100ml (McDowell 1989) so if plasma levels do not change then there will be no change in urinary excretion.

Previous studies (Snow, 1990; McMeniman and Hintz, 1992; White *et al*, 2001) have found that plasma ascorbate did not change as a result of exercise.

In addition, the method used was not particularly sensitive and vitamin analysis using HPLC would be preferred.

As well as measuring ascorbic acid it would have been useful to determine whether or not the horses were retaining the vitamin E supplements. The major route for vitamin E excretion is through the faeces, so faecal vitamin E content should also be established.

5.4 FUTURE WORK

- Establish the relationship between sweat TBARS and plasma TBARS and establish whether MDA is present in sweat using more selective methods such as HPLC.
- Establish the relationship between serum and saliva DPPH activities in the horse.
- Establish how sweat TBARS and DPPH activity in saliva changes with exercise. This may prove difficult, especially for sweat as it is nearly impossible to collect

sweat from a resting horse, unless sweating is induced by heat, fear or chemical means.

- Investigate the effect of season on the response of working horses to antioxidant supplementation.
- Investigate the link between body condition and oxidative stress, particularly with regard to TBARS.
- Investigate the effects of a range of antioxidants. The results from chapter 4 show little response to treatment with vitamin E. This may be due to many factors relating to the trial, but possibly supplementation with just one antioxidant is not the most effective method. Vitamin E is a fat-soluble vitamin and does not contribute to free radical scavenging activity in the aqueous environment of the cell. Antioxidants have complex interactions between each other such as the interactions between vitamin E, ascorbic acid and β carotene. Lowe (2002) suggests that studies examining the way in which combinations of classical dietary antioxidants and key plant components can be combined to enhance the total antioxidant defence system of the animal have shown improvements in total antioxidant capacity. Plants contain many antioxidants, including carotenoids and polyphenolic bioflavenoids. Preliminary studies have shown that milk thistle and dandelion decrease levels of urinary TBARS in horses following supplementation. Controlled studies involving supplementation with plants as well as other antioxidants would be useful.

5.4.1 Urate

Rasanen *et al* (1996) measured plasma concentration of uric acid, total peroxyl radical-trapping antioxidant parameter (TRAP) and plasma activity of xanthine oxidase in horses following bouts of exercise with increasing intensity. Exercise caused an increase in TRAP and in the concentration of uric acid. Plasma xanthine oxidase activity increased during exercise but intensity had only a minor effect. Mills *et al* (1997) investigated the effect of allopurinol (a XO inhibitor) administration to horses performing intense work. Significantly higher levels of hypoxanthine and xanthine were observed in the plasma of horses that received allopurinol than in control horses and in control horses plasma uric acid was significantly higher. In addition, allopurinol significantly reduced lipid hydroperoxides, oxidised glutathione and the glutathione redox ratio. Measurement of uric acid therefore is a good indicator of oxidative stress. However, the presence of the enzyme uricase in horses means that uric acid is converted to allantoin. Measurement of allantoin in urine or urate/allantoin ratio may be an effective measurement of oxidative stress during exercise.

Uric acid is present at high levels in serum of humans relative to other animals due to the functional depletion of urate oxidase and it has been suggested that urate is an important free radical scavenger *in vivo* (Hellsten *et al* 1997). Hellsten *et al* (1997) demonstrated that urate was oxidised to allantoin in human skeletal muscle during exercise, probably due to the generation of free radicals. Mikami *et al* (2000) found that after exercise at VO2 max in humans, urinary allantoin excretion significantly increased during 60 min of recovery but urinary urate excretion decreased significantly during the same period. Urinary TBARS significantly increased during

the latter half of the recovery period. They suggest that allantoin is a reliable indicator of *in vivo* oxidative stress. This would be useful in humans as allantoin excretion in urine would normally be low. In horses, the presence of allantoin in urine would be normal, but changes in excretion during exercise may be a useful indicator of oxidative stress.

5.4.2 Iron homeostasis

One of the damaging effects of the superoxide radical is that it oxidises exposed [4Fe-4S] clusters in certain vulnerable enzymes causing the release of iron and enzyme inactivation. The "free" iron then reduces hydroperoxides to hydroxyl or alkoxyl radicals. Because the "free" iron will preferentially bind to anionic polymers, such as nucleic acids, or to anionic surfaces, such as cell membranes, these radicals will be generated adjacent to these targets and will preferentially attack them.

Mills *et al* (1996) found that prolonged exercise in horses significantly decreased the iron (Fe)-binding antioxidant activity of plasma and increased total plasma Fe levels. Measurement of "free" iron in plasma therefore may be a good indicator of oxidative stress. However, this is more difficult if a non-invasive approach is to be taken. Animals have a limited capacity to excrete iron and little is excreted in urine. However, it is possible that "free" iron may be detected in saliva or in sweat samples.

5.5 AGEING

The results from chapter 3 are consistent with increased lipid peroxidation in older horses.

The population of horses was divided into three age groups, namely, group 1 with an age range of 5-15 years and a mean age of 9.7 years; group 2 with an age range of 16-24 years and a mean age of 20.7 years; group 3 with an age range of 25-38 years and a mean age of 28.6 years. Group 1 had significantly lower levels of urinary TBARS compared with the other two groups ($p<0.05$) and there was no difference between groups 2 and 3. This suggests that horses above the age of 15 years show significantly increased lipid peroxidation.

Kasapoglu and Ozben (2001) measured thiobarbituric acid reactive substances in serum of 100 healthy humans in five age groups: 20-29; 30-39; 40-49; 50-59 and 60-69 years (this would correspond roughly to 6-9; 10-13; 13.5-16; 16.5- 19.5 and 20-23 years in horses). Carbonyl content in oxidatively modified proteins, serum vitamin E concentrations, serum vitamin C concentrations, enzymic activities of super-oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), were also determined.

The results showed that there was an age-related increase in the concentration of lipid peroxides. The mean MDA values were significantly higher in the age group 50-59 compared with that of 30-39 age group. Protein carbonyl content increased in an age-related pattern with significantly higher levels in the 60-69 age group compared to the 20-29 and 30-39 age groups. SOD activity was significantly higher in age groups 50-59 and 60-69 and CAT levels were significantly higher in

the 60-69 group compared to the 20-29 group. GPX activity decreased with ageing and vitamin C and E levels did not change with age.

These data and others (Leeuwenburgh 1994; Bhagwat 1997; Tokunaga *et al* 1998) support the theory that the level of oxidative stress increases during the ageing process. However, there is lack of agreement in the literature as to whether this is due to a decline in antioxidant defences or an increase in the rate of pro-oxidant generation.

A positive correlation between tissue concentrations of specific non-enzymatic antioxidants (e.g. Vitamin E and C) and the inherent life span of animals has been elucidated as well as with the enzymatic antioxidant superoxide dismutase (SOD) (Cutler *et al* 1991). However, Sohal and Orr (1992) suggest that following a comparison of antioxidant defence mechanisms in six different mammalian species, that there was not a clear association between these defences and maximum life span potential (MLSP) of the species. In contrast, mitochondrial rates of $O_2^{\bullet-}$ and H_2O_2 formation were found to increase with age and the MLSP was found to be inversely correlated with liver mitochondrial rates of $O_2^{\bullet-}$ and H_2O_2 generation.

Kasapoglu *et al* (2001) found that ageing was linked to an increase in the antioxidant enzymes SOD and CAT and suggests that the ageing process cannot be explained due to a lack of protection due to a decrease in the activity of antioxidant enzymes. However other studies have shown that ageing is associated with declined capacities of both enzymic and non-enzymic antioxidant systems in organs such as liver, brain, heart and kidney (Ji, 1993; Matsuo, 1993).

Leeuwenburgh *et al* (1994) found that the activities of SOD, CAT, GPX, GR (glutathione reductase), glutathione transferase, and glutamyltranspeptidase in

soleus and vastus lateralis muscle were significantly elevated with age in rats. Lipid peroxidation was also significantly increased, despite elevated levels of antioxidant enzymes. It was suggested that increased oxidative stress to senescent muscle caused by leakage of superoxide from mitochondria causes adaptation of cellular defence mechanisms.

Extensive animal studies on ageing demonstrate that caloric restriction slows the rate of ageing. It has been suggested that this is due to an associated decrease in oxygen free radicals produced by the mitochondria. Lass *et al* (1998) compared protein and lipid oxidative damage to upper hindlimb skeletal muscle mitochondria in mice fed ad libitum and those restricted to 40% fewer calories. Concentration of TBARS significantly increased in ad libitum fed rats as did levels of protein carbonyls. The rate of superoxide anion radical generation also increased in the ad libitum fed rats. No such changes were observed in the calorie restricted rats and life span was increased in calorie restricted rats by approximately 30-40%. Lopez-Torres *et al* (2002) found that long term caloric restriction significantly decreased H_2O_2 production in rat liver mitochondria and significantly reduced oxidative damage to mitochondrial DNA. It was noted that the caloric restricted mitochondria released less ROS per unit electron flow.

The data available regarding the link between glucose and insulin metabolism and oxidative stress (Preuss, 1997, Facchini *et al*, 2000, Ceriello, 2000) supports the view that increased oxidative stress in ageing animals is associated with increased free radical generation.

The horses used in the current study were clinically healthy and were still active, so does the fact that older horses have higher levels of lipid peroxidation really matter?

Would decreasing the amount of free radical damage through antioxidant supplementation increase life-span or quality of life? Many horses do not reach such mature ages as seen in the current study, indeed, some horses exhibit significant signs of ageing at 15 years. Genetic background obviously has an effect – ponies, on the whole are longer-lived than horses and certain breeds of horses such as Arabians have a reputation for longevity. Other variables such as activity, nutrition, management, and temperature can affect life-span, making it a highly complex multi-factorial process (Kasapoglu and Ozben 2001).

A recent study by Heaton *et al* 2001 investigated the effect of a combined antioxidant supplement on antioxidant, immunological and DNA parameters in adult dogs. Dogs (n=40) that had been maintained on the same diet were split into 2 groups of 20. One group received an antioxidant cocktail as a supplement for a period of 3 months. The results showed that dogs receiving the supplement had significantly higher levels of plasma taurine and a significant increase in total antioxidant potential in plasma. Taurine is suggested to stabilise the plasma membrane and prevent oxidant-induced increases in membrane permeability (Read and Welty 1963). Taurine has also been shown to act as an antioxidant protecting rat alveolar macrophages from oxidant damage (Banks *et al* 1992). Also, the dogs receiving no supplement had significantly higher levels of DNA damage present in leukocytes, as measured by the comet assay, than those dogs receiving the supplement. The authors concluded that dietary antioxidants could ameliorate DNA damage in cats and dogs.

Arivazhagan *et al* (2000) found that lipid peroxide levels in the blood of rats increased with age and that supplementation with dl-alpha-lipoic acid decreased

lipid peroxide levels. Other studies (Hagen *et al* 1999; Arivazhagan *et al* 2001) have found that supplementation with dl-alpha-lipoic acid decreased the level of mitochondrial lipid peroxidation and increased antioxidant status in liver and kidney mitochondria of aged rats.

One important factor to consider is the fact that horses in their teens are found in the highest levels of competition and many older horses remain active well into their twenties and sometimes thirties. The rate of oxygen consumption during exercise increases and O_2 flux in an active muscle may increase 100 - fold (Sen 1995), which can lead to increased production of free radicals.

It has been suggested that senescent organisms are more susceptible to oxidative stress during exercise and that ageing also increases the risk of muscle injury and the inflammatory response can subject senescent muscle to further oxidative stress (Ji 2001).

Malinowski and McKeever (1997) measured the exercise capacity of young (mean age 5 years) vs old (mean age 22 years) Thoroughbred and Standardbred mares. They concluded that older mares had a substantially lower maximal aerobic capacity, which corresponds to the fact that maximal heart rate also declines with age in horses, similar to the decrease observed in cardiovascular function in humans. In humans noticeable changes are also seen in aerobic capacity between 40 and 50 years but these parameters have not yet been established in horses (Malinowski and McKeever 1997).

Owners may continue to train their older animals by use of exercise regimes that although appropriate for younger horses may not be appropriate for older horses (Malinowski and McKeever 1997). A result may be an increased risk of injury and

so increased oxidative stress to the senescent muscle. Leeuwenburgh *et al* (1994) showed that there was a significant increase in lipid peroxidation in ageing muscle, despite elevated levels of antioxidant enzymes. Meydani and Evans (1993) found that vitamin E supplementation was more effective in preventing muscle injury in old subjects than in young ones.

For working senior horses, therefore, supplementation with antioxidants may be beneficial. An increased susceptibility to infections is also observed in older horses (Ralston 2002) so supplementation with antioxidants such as vitamin C and vitamin E may also improve immune function as well as reducing oxidative damage to tissues.

Urinary analysis using ^{1}H NMR spectroscopy and proteinuria quantification using the biuret assay demonstrated that horses showed both increased total proteinuria and increased excretion of aromatic amino acids with increasing age. This could suggest that subtle changes in renal, hepatic and endocrine functions are also evident in senior horses. Increased TBARS excretion in urine and increased proteinuria in senior horses may suggest that increased oxidative stress to ageing tissues may cause glomerular injury, thus leading to increased protein excretion. In addition, administration of antioxidants to animals suffering from PAN induced nephrosis, decreased proteinuria and ameliorated damage to glomerular epithelial cell foot processes (Ricardo *et al* 1994). Rations for senior horses, therefore, should possibly contain higher levels of antioxidants and although renal disease may not necessarily be apparent in senior horses it may be sensible to also provide a ration which is supportive of the renal system. In addition, horses have been observed to

accumulate cadmium in the kidneys (Penumarthy *et al*, 1980, Salisbury *et al*, 1991, Baldini *et al*, 2000), which could cause proximal tubular damage. Horses excrete excess dietary calcium through their urine instead of faeces, as do other animals, so high calcium ingredients such as alfalfa, and beet pulp should be avoided. Protein and phosphorus should be restricted to 8 –12% and 0.25% respectively (Ralston 2002), therefore wheat or rice bran, or high levels of cereals should also be avoided because of a high phosphorus content. Good quality grass, grass hay or haylage intake should be maximised.

In addition, data from NMR spectra suggest increased protein catabolism which may be consistent with pituitary dysfunction. Old mares with pituitary dysfunction, even in the early pre-clinical stages had lower blood vitamin C than did unaffected or younger mares (Ralston 2002). Therefore, supplementation with this particular antioxidant may be advisable for senior horses. In addition, horses with this condition become relatively glucose intolerant so high fibre and high fat diets with limited soluble carbohydrate (i.e. limited molasses) may help to control this problem.

5.6 FUTURE WORK

In this study the samples were obtained on a random basis – from horses owned by members of the public in response to adverts placed in popular equine magazines. As a result there was little control over the population of horses used. The results are a preliminary examination of an equine population to establish whether there is increased free radical damage, as assessed by lipid peroxidation, in older horses. A natural progression would be to carry out a more stringently controlled trial. Ideally, horses housed in the same yard, under the same management routine, eating the same diet would be used. However, it is extremely difficult to obtain sufficient number of samples from horses of such diverse age groups. Also, obviously, horses who are in their twenties and thirties would have very different life histories than horses who are five or six, for example.

Dividing the horses into narrower age groups would be useful, for example, using age groups similar to the ones used in the study carried out by Kasapoglu and Ozben (2001) in humans. This would allow a more accurate picture of when changes due to free radical damage occur in the horse to emerge.

The current study used urinary TBARS to investigate oxidative stress and ^{1}H NMR spectroscopy and total urinary protein to investigate other age-related changes. To further confirm the effect of oxidative stress during ageing and to investigate whether oxidative stress occurs due to increased pro-oxidant generation or decreased antioxidant defences, antioxidant status should also be determined as well as using further markers of oxidative stress.

The activity of antioxidant enzymes in serum or plasma could be measured, for example, glutathione peroxidase, glutathione reductase, superoxide dismutase and

catalase. Also serum or plasma levels of specific antioxidants could be determined, for example, vitamin C or vitamin E. However, it may not be useful to just measure a single or a combination of just a few antioxidants as a decrease in one antioxidant can be compensated by an increase in a different one (Kasapoglu and Ozben 2001). It may be more useful to concentrate on measurement of total antioxidant capacity which gives an overall indication of the antioxidant status of an individual. There are many published methods, for example, Koracevic *et al* (2001) described a method which measured the capacity of a biological fluid to inhibit the production of TBARS from sodium benzoate under the influence of free oxygen radicals derived from the Fenton reaction. The method of Atsumi *et al* (1999) described in chapter two is based on the reduction rate of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). A commercially available assay (Randox Laboratories Ltd, Co Antrim) uses 2,2'-Azino-di [3-ethylbenthiazoline sulphate] (ABTS). This is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{\bullet+}$. This has a relatively stable blue-green colour measured at 600nm. Antioxidants in the added sample cause suppression of this colour production to a degree which is proportional to their concentration.

The Trolox equivalent antioxidant capacity assay (TEAC) assesses the activity of antioxidants by comparing their activities with that of Trolox (a vitamin E analogue) for scavenging a free radical. As well as assessing the ability to scavenge the radical, the delay of radical generation is determined and the delay in oxidation is used as a parameter for the antioxidant activity (Schlesier *et al* 2002).

The Ferric reducing ability of plasma assay (FRAP assay) measures the ability to reduce the ferric ion. Ferric to Ferrous ion reduction at low pH causes a coloured

ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance changes at 593nm in test reaction mixtures with those containing ferrous ions in known concentrations.

If a non-invasive approach is to be taken other urinary markers can be measured such as F2 isoprostanes. These appear to be an extremely useful non-invasive biomarker of oxidative stress and can now be measured in urine, without extensive pretreatment, using an ELISA assay. The ELISA assay has been validated for use in human urine and there is strong correlation between values obtained by GC-MS and ELISA. This would need to be established for equine urine and used alongside other markers of oxidative stress to establish its value as a non-invasive marker for horses. The use of free radical scavenging activity of saliva could also be further developed to investigate any potential changes with age. A lifelong study to investigate any potential effects of antioxidant supplementation on life span, incidence of disease, and general activity could also be undertaken. In practice this would be very difficult, although the concept is attractive. It would be more useful to concentrate on the requirements of working older horses and to further establish how their response to exercise differs from younger horses and if as a result their nutrient requirements are different. A controlled exercise test investigating senior horses' response to exercise following antioxidant supplementation may also be beneficial. A recent study (Williams *et al* 2002) supplemented five thoroughbred geldings with 10mg/kg/d dl-alpha-lipoic acid. Red and white blood cells and plasma were analysed for glutathione, glutathione peroxidase, and total plasma lipid hydroperoxides, at baseline, after 7 and 14 days of supplementation and 48 hours postsupplementation. Plasma lipid hydroperoxides were lower in the supplemented

group compared to a control group and glutathione peroxidase activity was seen to be lower in the supplemented group. The authors concluded that 10mg/kg dl alpha lipoic acid had no evident adverse effects and moderately reduced the oxidative stress of horses allowed light activity. These findings along with the reported beneficial effects of dl-alpha- lipoic acid on aged rats suggest that this supplement may be of use in senior horses and particularly for working older horses, and further studies involving this supplement should be undertaken.

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Internet Resources

http://crystal.uah.edu/~carter/enzyme/catalase

http://fmrc.pulmcc.washington.edu/DOCUMENTS/FMRC299.pdf/

http://members.tripod.com/

http://morgan.rutgers.edu/HTMLdocs/physics_modules/Magnetic_Resonance

www.beta-uk.org

www.bhb.co.uk

www.dodsonandhorrell.com

www.isat.jmu.edu/users/klevicca/isat454/NMRChemscape.doc

www.srs.dl.ac.uk/mbg/sod/

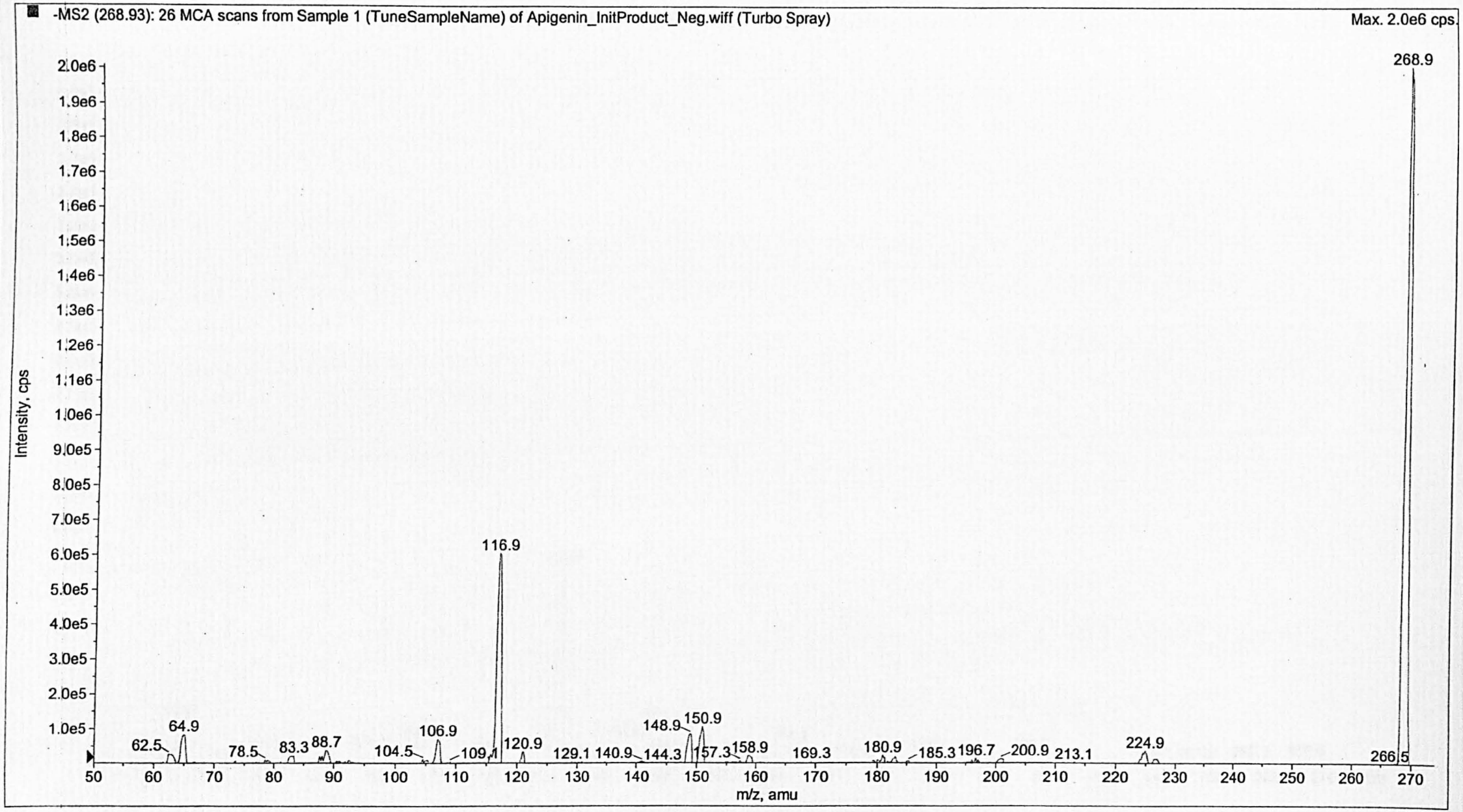
www.waltham.com

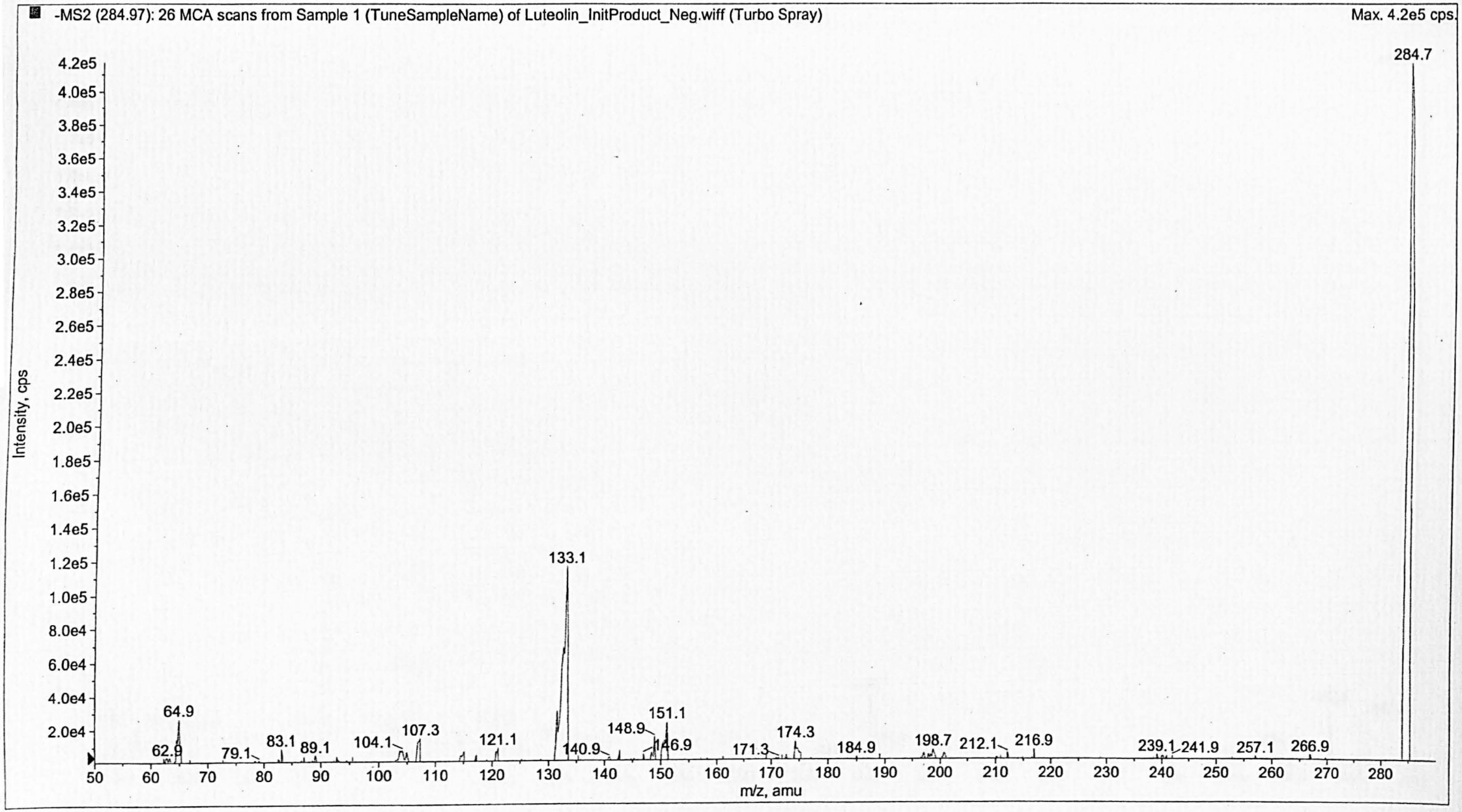
www.worthington-biochem.com/manual/s/SOD/

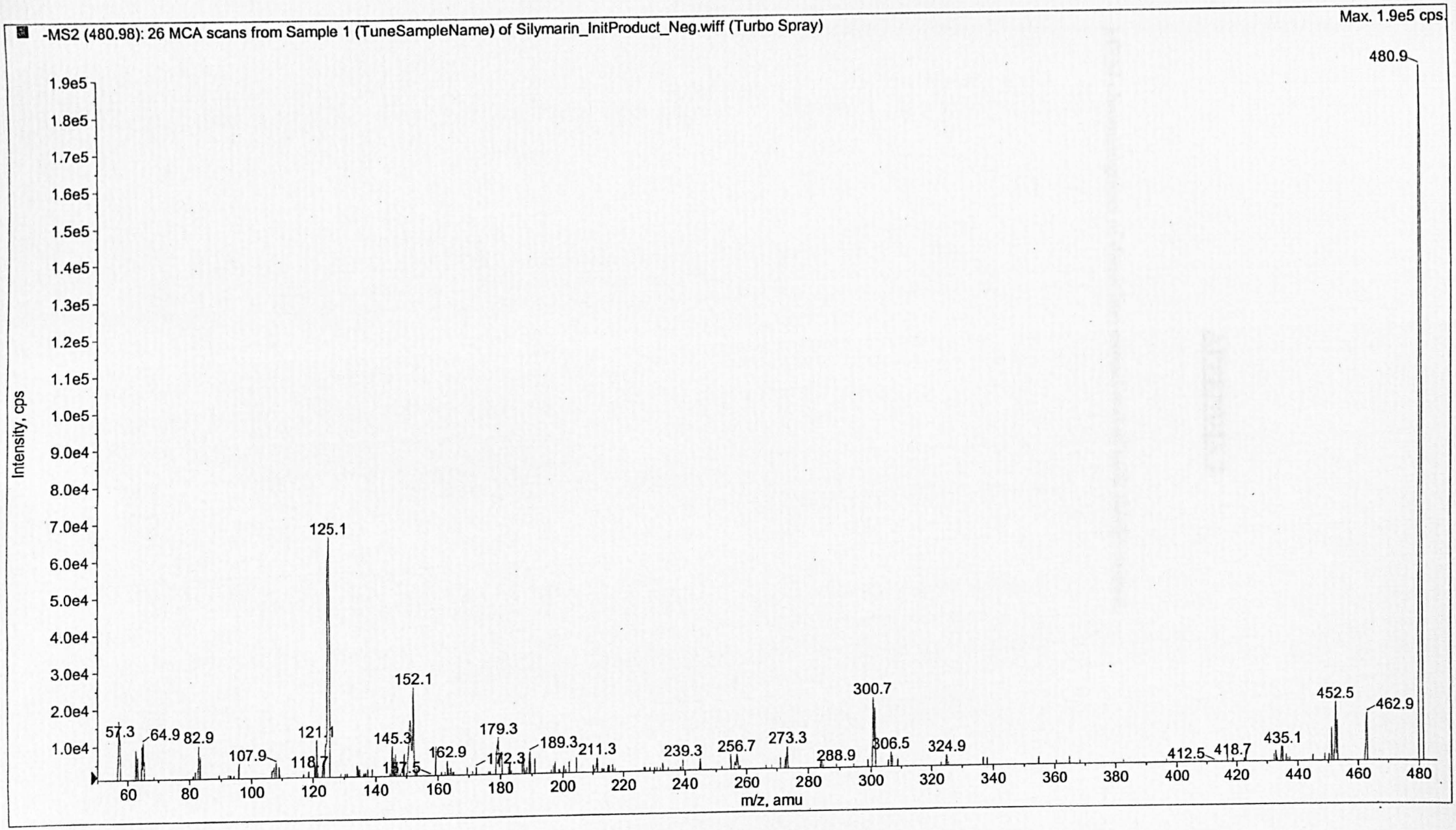
APPENDICES

APPENDIX ONE

Negative MS/MS product ion scan for apigenin, lueolin and silymarin, respectively.


-MS2 (268.93): 26 MCA scans from Sample 1 (TuneSampleName) of Apigenin_InitProduct_Neg.wiff (Turbo Spray)
Max. 2.0e6 cps.
Intensity, cps
m/z, amu
268.9
116.9
64.9
106.9
150.9
148.9
62.5
78.5
83.3
88.7
104.5
109.1
120.9
129.1
140.9
144.3
157.3
158.9
169.3
180.9
185.3
196.7
200.9
213.1
224.9
266.5


-MS2 (284.97): 26 MCA scans from Sample 1 (TuneSampleName) of Luteolin_InitProduct_Neg.wiff (Turbo Spray)
Max. 4.2e5 cps.
Intensity, cps
m/z, amu
284.7
133.1
151.1
64.9
62.9
79.1
83.1
89.1
104.1
107.3
121.1
140.9
148.9
146.9
171.3
174.3
184.9
198.7
212.1
216.9
239.1
241.9
257.1
266.9


-MS2 (480.98): 26 MCA scans from Sample 1 (TuneSampleName) of Silymarin_InitProduct_Neg.wiff (Turbo Spray)
Max. 1.9e5 cps.
Intensity, cps
m/z, amu
480.9
125.1
152.1
300.7
452.5
462.9
57.3
64.9
82.9
107.9
121.1
118.7
145.3
157.5
162.9
179.3
172.3
189.3
211.3
239.3
256.7
273.3
288.9
306.5
324.9
412.5
418.7
435.1

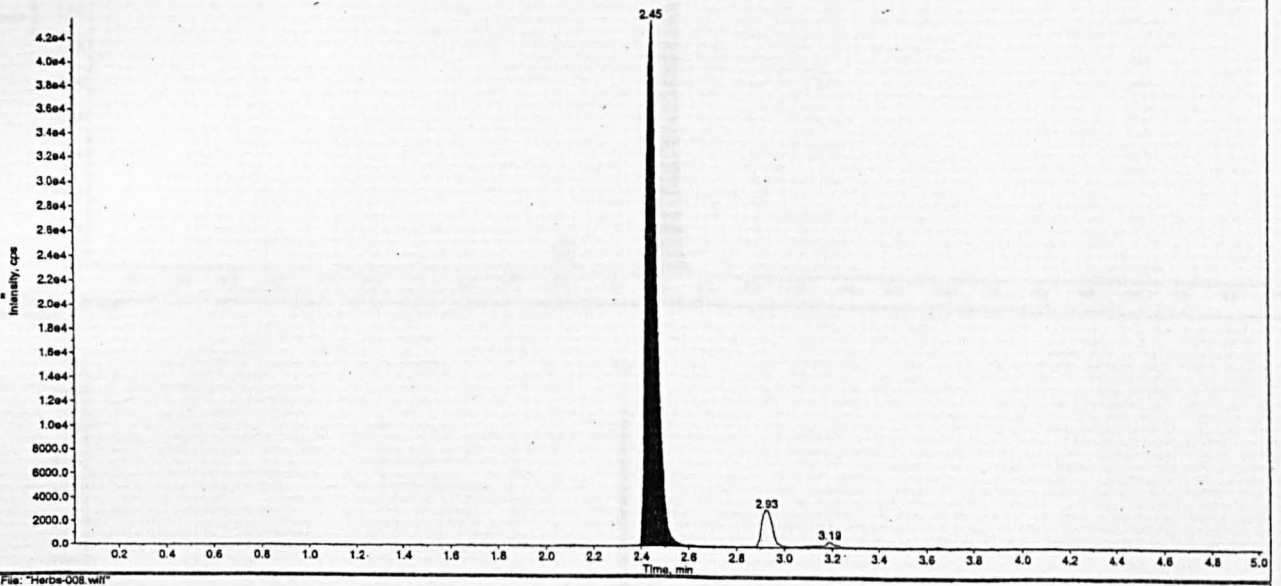
APPENDIX 2

MRM chromatograms of dandelion extract and of milk thistle extract.

roject: Herbs Results Name: Herb extracts.rdb Analyst Version: 1.3.1

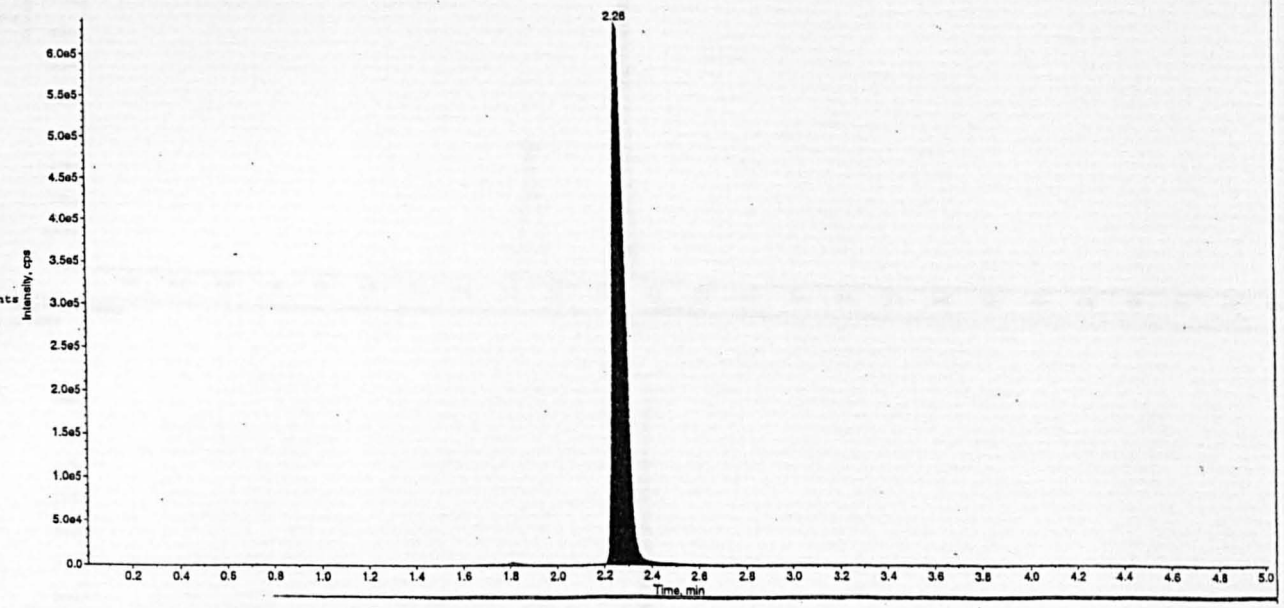
Sample Name: "Dandelion" Sample ID: "" File: "Herbs-008.wiff"
Peak Name: "Apigenin" Mass(es): "268.9/117.1 amu"
Comment: "" Annotation: ""

ample Index:	1
ample Type:	Unknown
oncentration:	N/A
alculated Conc:	73.0 ng/mL
eq. Date:	14/06/2003
eq. Time:	16:15:57
odified:	No
unching Factor:	2
oise Threshold:	106.96 cps
rea Threshold:	534.82 cps
um. Smooths:	0
T Window:	30.0 sec
xpected RT:	2.44 min
ep. Width:	0.20
ep. Height:	0.01
xp. Peak Ratio:	5.00
xp. Adj. Ratio:	4.00
xp. Val. Ratio:	3.00
se Relative RT:	No
Int. Type:	Base To Base
Retention Time:	2.45 min
Area:	136727.540 counts
ight:	43900. cps
Start Time:	2.36 min
End Time:	2.63 min



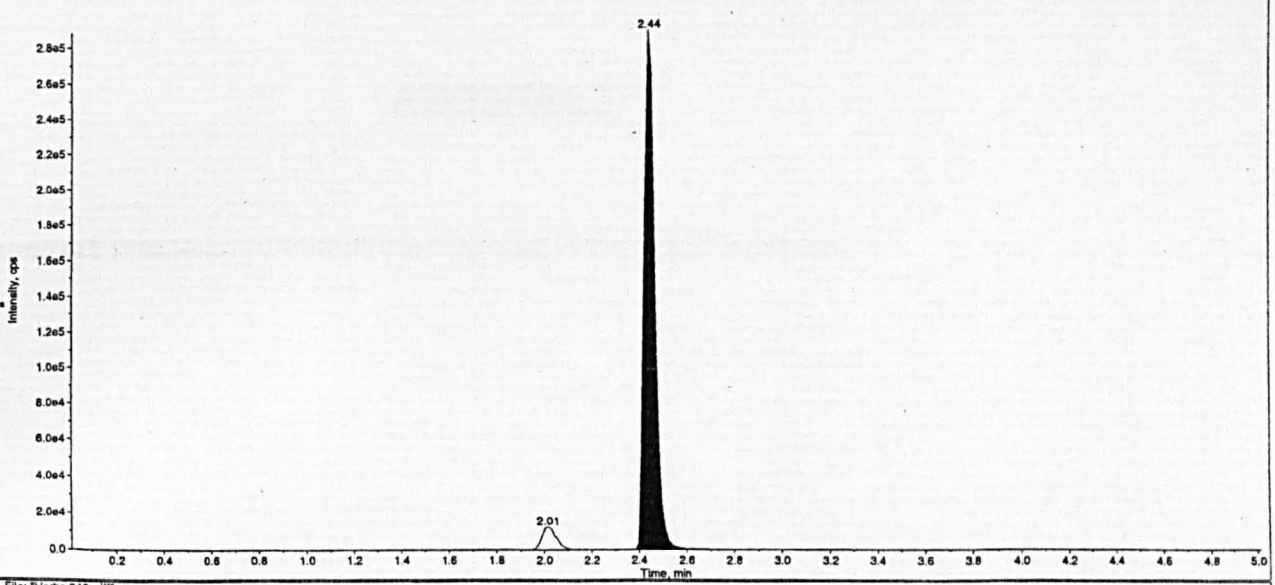
Sample Name: "Dandelion" Sample ID: "" File: "Herbs-008.wiff"
Peak Name: "Luteolin" Mass(es): "285.0/133.1 amu"
Comment: "" Annotation: ""

Sample Index:	1
Sample Type:	Unknown
Concentration:	N/A
Calculated Conc:	2450.2 ng/mL
Acq. Date:	14/06/2003
Acq. Time:	16:15:57
Modified:	No
Bunching Factor:	2
Noise Threshold:	65.74 cps
Area Threshold:	328.72 cps
Num. Smooths:	0
RT Window:	30.0 sec
Expected RT:	2.27 min
Sep. Width:	0.20
Sep. Height:	0.01
Exp. Peak Ratio:	5.00
Exp. Adj. Ratio:	4.00
Exp. Val. Ratio:	3.00
Use Relative RT:	No
Int. Type:	Base To Base
Retention Time:	2.26 min
Area:	2243011.900 counts
Height:	665000. cps
Start Time:	2.19 min
End Time:	2.45 min



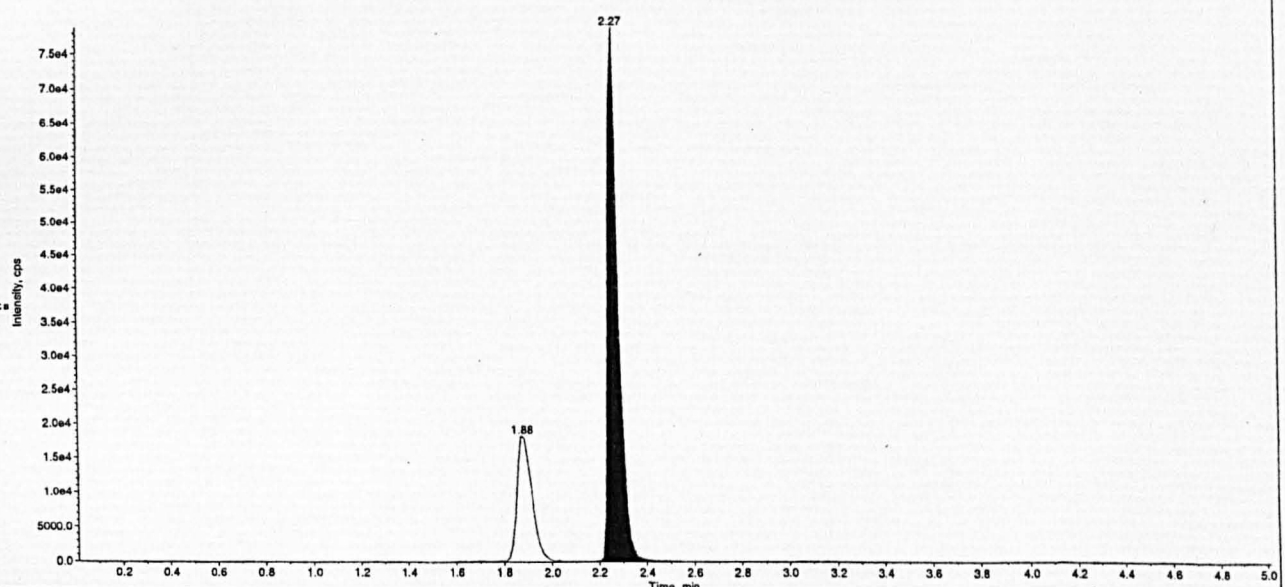
Sample Name: "Milk Thistle" Sample ID: "" File: "Herbs-010.wiff"
Peak Name: "Apigenin" Mass(es): "268.9/117.1 amu"
Comment: "" Annotation: ""

Sample Index:	1
Sample Type:	Unknown
Concentration:	N/A
Calculated Conc:	572.4 ng/mL
Acq. Date:	14/06/2003
Acq. Time:	16:26:49
Modified:	No
Bunching Factor:	2
Noise Threshold:	106.96 cps
Area Threshold:	534.82 cps
Num. Smooths:	0
RT Window:	30.0 sec
Expected RT:	2.44 min
Sep. Width:	0.20
Sep. Height:	0.01
Exp. Peak Ratio:	5.00
Exp. Adj. Ratio:	4.00
Exp. Val. Ratio:	3.00
Use Relative RT:	No
Int. Type:	Base To Base
Retention Time:	2.44 min
Area:	916171.122 counts
Height:	296000. cps
Start Time:	2.36 min
End Time:	2.70 min



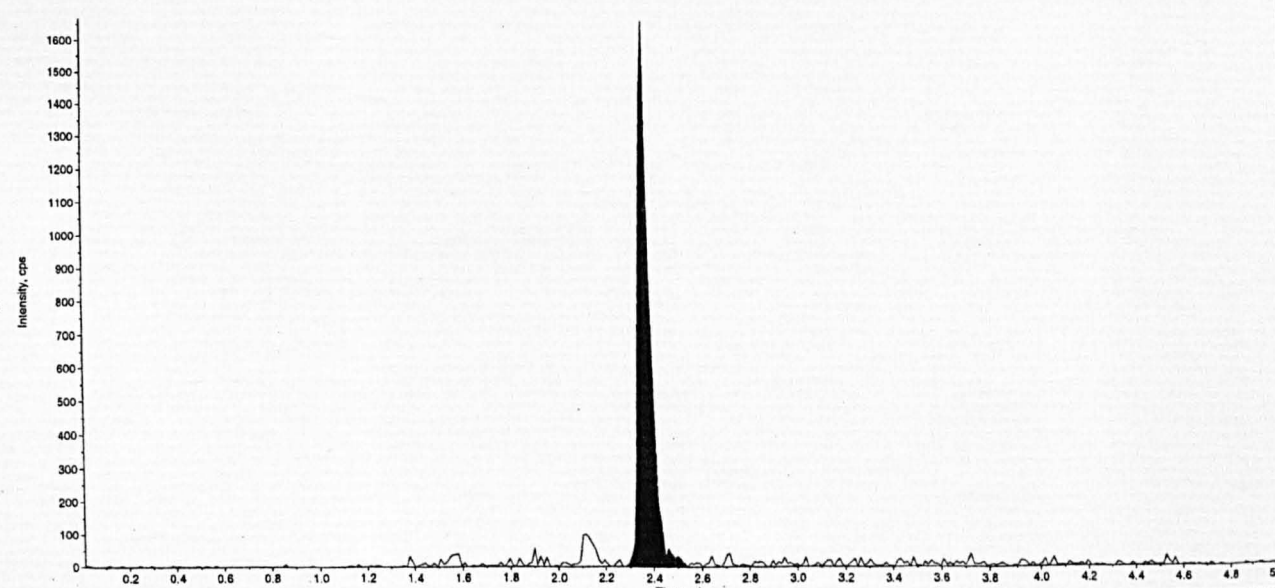
Sample Name: "Milk Thistle" Sample ID: "" File: "Herbs-010.wiff"
Peak Name: "Luteolin" Mass(es): "285.0/133.1 amu"
Comment: "" Annotation: ""

Sample Index:	1
Sample Type:	Unknown
Concentration:	N/A
Calculated Conc:	272.8 ng/mL
Acq. Date:	14/06/2003
Acq. Time:	16:26:49
Modified:	No
Bunching Factor:	2
Noise Threshold:	65.74 cps
Area Threshold:	328.72 cps
Num. Smooths:	0
RT Window:	30.0 sec
Expected RT:	2.27 min
Sep. Width:	0.20
Sep. Height:	0.01
Exp. Peak Ratio:	5.00
Exp. Adj. Ratio:	4.00
Exp. Val. Ratio:	3.00
Use Relative RT:	No
Int. Type:	Base To Base
Retention Time:	2.27 min
Area:	242599.576 counts
Height:	78900. cps
Start Time:	2.16 min
End Time:	2.51 min



Sample Name: "Milk Thistle" Sample ID: "" File: "Herbs-010.wiff"
Peak Name: "Silymarin" Mass(es): "481.0/125.1 amu"
Comment: "" Annotation: ""

Sample Index:	1
Sample Type:	Unknown
Concentration:	N/A
Calculated Conc:	32.8 ng/mL
Acq. Date:	14/06/2003
Acq. Time:	16:26:49
Modified:	No
Bunching Factor:	2
Noise Threshold:	17.91 cps
Area Threshold:	89.54 cps
Num. Smooths:	0
RT Window:	30.0 sec
Expected RT:	2.36 min
Sep. Width:	0.20
Sep. Height:	0.01
Exp. Peak Ratio:	5.00
Exp. Adj. Ratio:	4.00
Exp. Val. Ratio:	3.00
Use Relative RT:	No
Int. Type:	Base To Base
Retention Time:	2.37 min
Area:	5677.414 counts
Height:	1690. cps
Start Time:	2.28 min
End Time:	2.54 min



APPENDIX 3

Questionnaire sent to horse owners participating in the senior horse trial.

DE MONTFORT UNIVERSITY - SENIOR HORSE PROJECT

YOUR NAME & ADDRESS

NAME OF HORSE

AGE OF HORSE

SEX AND BREED

BRIEF HISTORY

WHAT IS THE DAILY RATION FOR YOUR HORSE?

IS YOUR HORSE RECEIVING ANY SUPPLEMENTS? – PLEASE DETAIL

IS YOUR HORSE ON ANY MEDICATION? – PLEASE DETAIL

WHAT IS YOUR HORSE'S EXERCISE ROUTINE?

WAS YOUR HORSE WORKED BEFORE COLLECTION OF THE URINE SAMPLE? IF YES PLEASE SPECIFY.

DATE & TIME (APPROX) THE URINE SAMPLE WAS COLLECTED?

APPENDIX 4

Horse	D.O.B	Sex	Height (hh)	Weight (kg)
1	1987	gelding	17	622
2	1986	mare	16.3	616
3	1989	gelding	16.2	566
4	1987	gelding	16.2	588

Concentrate ration (grams x3 per day)

Horse	Hunt event cubes	Quiet Pencils	Old Faithfuls	Weight gain
1	100	0	0	0
2	0	700	0	500
3	0	100	0	0
4	0	0	100	0

Vitamin E content of concentrate feed (mg/kg)

Hunt event cubes	160
Quiet pencils	80
Old Faithfuls	100
Weight gain	100

Vitamin E supplement received at 4mg/kg bodyweight

Horse	αtocopherol acetate (g)
1	2.48
2	2.46
3	2.26
4	2.35

APPENDIX 5

Horse	Age (years)	Breed	Sex	Height (hh)	Weight (Kg)
Barty	20	TB	Gelding	16.1	578
Stamp	15	TB	Gelding	17.2	626
Scotty	20	TB	Gelding	16.2	560
Salesman	15	TB	Gelding	16.0	550
Jerome	13	TB	Gelding	16.2	618
Buddy	15.1	TB	Gelding	15.1	522
Lloyd	13	TB	Gelding	16.2	590

Diets

Horse	Feed	Quantity (Kg)
Barty	Quiet Pencils	1.0
	Haylage	13
Stamp	Old Faithfuls	2.0
	Haylage	17
Scotty	Quiet Pencils	2.0
	Haylage	13
Salesman	Quiet Pencils	1.0
	Haylage	11
Jerome	Quiet Pencils	1.0
	Haylage	13
Buddy	Quiet Pencils	0.75
	Haylage	10
Lloyd	Quiet Pencils	0.5
	Haylage	15

Horses also received 2kg of HiFi (Dengie) and 2 scoops of sugarbeet at each feed.

Rations were supplemented with salt.

Chemical composition of concentrate feed

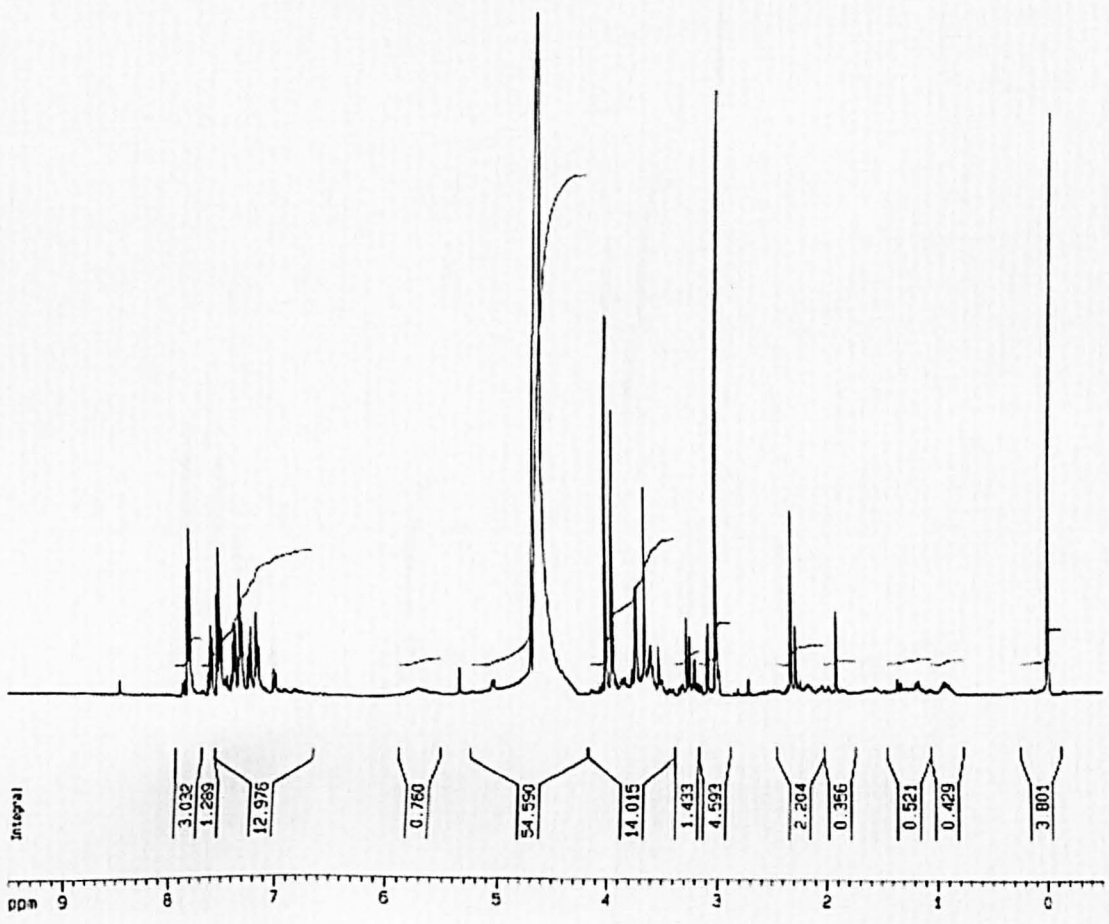
Nutrient	Quiet Pencils	Old Faithfuls
Moisture %	13.8	13.8
CP %	10	10
CF %	16	7
EE %	2.75	3
Ash %	10.5	7.5
Vitamin A IU	10,000	10,000
Vitamin D3 IU	1,500	1,500
Vitamin E IU	80	80
Cu mg/kg	17	15
GE MJ/kg	9	11

Vitamin E supplement received at 3mg/kg bodyweight

Horse	α-tocopherol acetate (g)
Barty	1.73
Stamp	1.88
Scotty	1.68
Salesman	1.65
Jerome	1.85
Buddy	1.57
Lloyd	1.77

APPENDIX 6

Horse 12 BRACKEN (20 years old)
WATERSUP H2O+D2O {C:\u] meedhan 3

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Current Data Parameters
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EXPNO                   108
PROCNO                    1

F2 - Acquisition Parameters
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PULPROG                zgpr
TD                    32768
SOLVENT             H2O+D2O
NS                         64
DS                          2
SWH                4844.961 Hz
FIDRES             0.147856 Hz
AQ                 3.3817077 sec
RG                        114
DW                  103.200 usec
DE                    6.00 usec
TE                   300.0 K
D1                 5.00000000 sec
d12                0.00002000 sec
d13                0.00000300 sec

--------- CHANNEL f1 ---------
NUC1                      1H
P1                     8.50 usec
PL1                    0.00 dB
PL9                   55.39 dB
SFO1            400.1318806 MHz

F2 - Processing parameters
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SF              400.1299961 MHz
WDW                       EM
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GB                         0
PC                      1.00

1D NMR plot parameters
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CY                     12.50 cm
F1P                    9.500 ppm
F1                  3801.24 Hz
F2P                   -0.500 ppm
F2                  -200.07 Hz
PPMCM                0.50000 ppm/cm
HZCM               200.06500 Hz/cm
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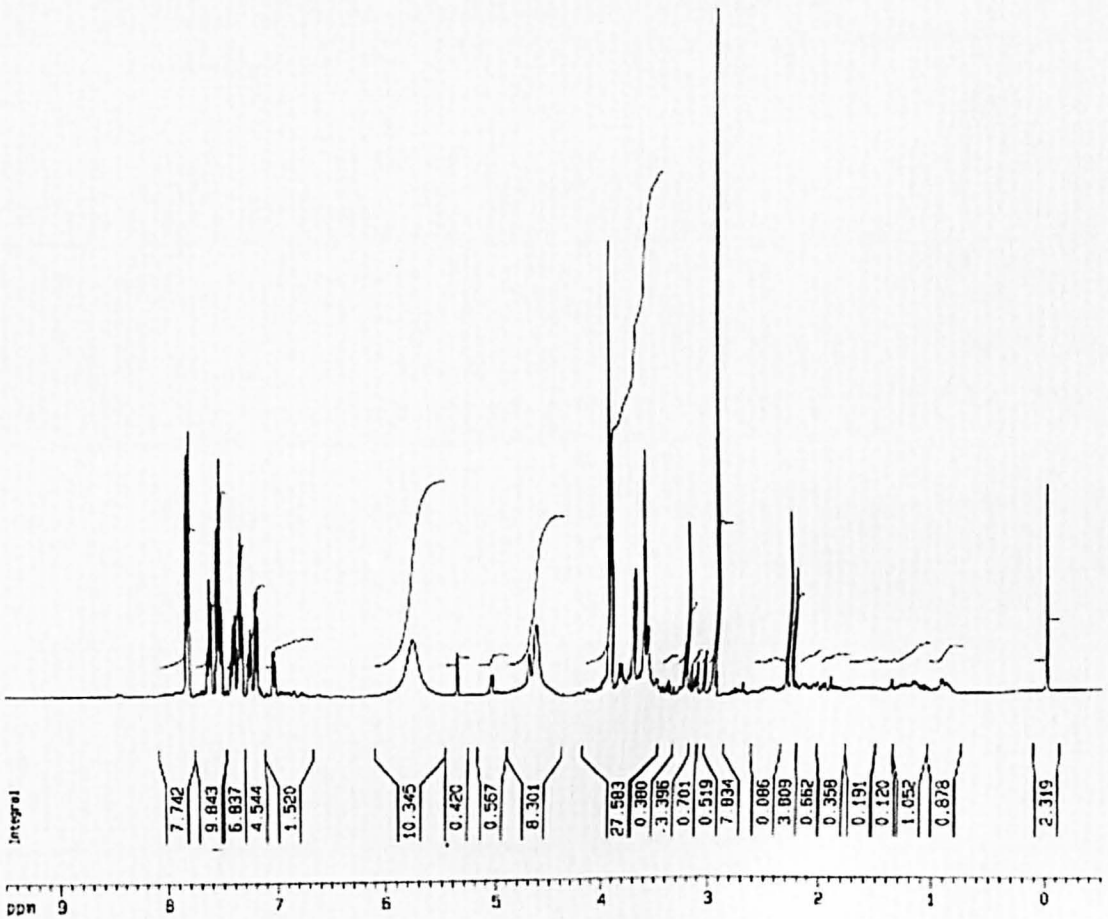
Horse 6 (5 years old)
WATERSUP H2O+D2O (C:\u) trevor 4



Current Data Parameters
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EXPNO 130
PROCNO 1

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Time 12.14
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PULPROG zgpr
TD 32768
SOLVENT H2O+D2O
NS 64
DS 2
SWH 4844.961 Hz
FIDRES 0.147856 Hz
AQ 3.3817077 sec
RG 71.8
DW 103.200 usec
DE 6.00 usec
TE 300.0 K
D1 5.00000000 sec
d12 0.00002000 sec
d13 0.00000300 sec

-------- CHANNEL f1 --------
NUC1 1H
P1 8.50 usec
PL1 0.00 dB
PL9 55.39 dB
SFO1 400.1318806 MHz

F2 - Processing parameters
SI 16384
SF 400.1299977 MHz
WDW EM
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 20.00 cm
CY 12.50 cm
F1P 9.500 ppm
F1 3801.24 Hz
F2P -0.500 ppm
F2 -200.07 Hz
PPMCM 0.50000 ppm/cm
HZCM 200.06500 Hz/cm